Research Thesis: Inhibition of the Glycoprotein Von Willebrand Factor via an RNA Aptamer in a Large Animal Model of Middle Cerebral Arterial Occlusion Stroke

Presented in partial fulfillment of the requirements for graduation *with Research Distinction* in *Biomedical Engineering* in the undergraduate colleges of The Ohio State University, 2021

Cole P. Anderson

Thesis Advisors: Dr. Shahid Nimjee, MD, PhD Associate Professor, Department of Neurological Surgery Wexner Medical Center, and Dr. Tonya Nocera, PhD Associate Professor-Clinical, Department of Biomedical Engineering at The Ohio State University

Table of Contents

For my grandfather

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less." – Marie Curie

I. Abstract

Every three and a half minutes an individual dies from a stroke. Approximately 85% of strokes are ischemic, and over half of all ischemic strokes originate in the middle cerebral artery (MCA)[1-2,6]. Currently, standard stroke treatment includes intravenous infusion of recombinant tissue plasminogen activator (rTPA), a serine protease that's primary function is to eliminate occlusion caused by blood clots via thrombolysis[3]. This method is only marginally effective at thrombolysis, cannot be reversed, and must be given within a timeframe of 3-4.5 hours of stroke onset, due to increased hemorrhage risk. Knowing that von Willebrand Factor (vWF), a known glycoprotein mediator in platelet adhesion and aggregation, plays a major role in vessel injury hemostasis, the Nimjee Lab developed a vWF-inhibiting aptamer, DTRI-031 (REF Oney, Nimjee et al. Oligonucleotides 2007; Nimjee et al. Molecular Therapy 2012 and Nimjee et al. Molecular Therapy 2019). Aptamers are RNA or DNA oligonucleotides which bind to and inhibit specific target molecules (REF Nimjee et al. Annual Reviews of Medicine 2005).

In this study, we tested the hypothesis that DTRI-031 can lyse a blood clot 6 hours after it is placed in the MCA of canine hounds[4]. Furthermore, DTRI-031 can be rapidly reversed by another single-stranded RNA ligand, DTRI-025, to mitigate hemorrhage risk induced by the activity of DTRI-031. This study aims to demonstrate the efficacy of DTRI-031 to thrombolyse an occlusive thrombus in a large animal MCA occlusion (MCAO) model by measuring functional differences at different aptamer concentrations, like reperfusion rate, infarct volume, and inhibition of platelet activity and aggregation. Additionally, this study aims to demonstrate the reversibility of DTRI-031 by DTRI-025 using a point-of-care platelet activity assay (PFA-100) and platelet aggregometry. We anticipate that higher concentrations of DTRI-031 will result in improved cerebral perfusion, decreased infarct size, and lower platelet aggregation. Moreover, DTRI-025

will rapidly and durably reverse DTRI-031 functionality in both platelet activity and aggregation assays.

II. Introduction

A. Historical Background, Motivation, and Significance:

According to the American Stroke Association (ASA) "Heart Disease and Stroke Statistics: 2021 Edition", approximately 920,000 Americans suffer a stroke annually. Of those, 795,000 are acute ischemic strokes. Approximately, 610,000 AIS are first-time strokes, and 185,000 are recurrent[6]. There are three main categories of stroke: acute ischemic stroke (AIS), intracerebral hemorrhagic stroke (ICH), and subarachnoid hemorrhagic stroke (SAH). Hemorrhagic strokes are defined by the rupture/tearing of blood vessels within the brain, leading to blood pooling inside the skull, whereas AIS presents via a blockage within the vessel itself, limiting oxygen to the brain. Looking again at the ASA "Heart Disease and Stroke Statistics: 2021 Edition", of all strokes, 87% are AIS, 10% are ICH, and 3% are SAH[6].

Currently, the prevailing treatments for AIS include antiplatelet and thrombolytic therapy. Antiplatelet therapies work to stop platelets from sticking together and forming a clot through the use of drugs like low-dose aspirin. While these drugs can be helpful in clot prevention, they have the potential to cause side effects like gastrointestinal bleeding or hemorrhagic stroke[7-9]. Thrombolytic therapies, on the other hand, work to break apart (lyse) an existing blood platelet clot. Currently, the only FDA approved thrombolytic therapy for the treatment of ischemic stroke is rTPA, which is only given to approximately 8.5% of patients who present with AIS. Additionally, >66% of US hospitals rarely, or never, administer rTPA to AIS patients due to the small timeframe in which it can be given (<4.5 hours) and potential harmful side effects, such as

hemorrhaging, that can present after its administration to the patient[10-11]. These side effects can also include additional stroke onset in the form of hemorrhagic stroke due to the rTPA thinning the blood in the process of lysing the thrombus, with no reversibility[12]. As a result, there is a distinct lack of safe, and reversible, therapeutic options for both the early and long-term treatment of AIS patients [3-5].

B. Platelet Aggregation and Coagulation:

Understanding the basic concepts of blood clot formation is a crucial first step in developing a safe and effective treatment for Ischemic stroke. As such, it is important to take the time and understand the role that platelets have in blood clot formation, one of the smallest factors involved in the formation of a thrombus. Platelets are small anuclear cell fragments that are produced by megakaryocytes in the bone marrow[13]. Under hemostatic conditions, platelets will circulate within vasculature, but not stick to the endothelial surface of vessels for an extended period of time. Once a vascular injury occurs, the endothelial cell layer is compromised and the subendothelial extracellular matrix (ECM) becomes exposed, creating a highly thrombogenic environment for platelet adhesion. To return to hemostasis, platelets will adhere to the ECM at the injured site and form a hemostatic plug. This is largely accomplished via interaction of the platelet membrane receptor complex, Glycoprotein Ib-IX-V (GPIb-IX-V), and the A1 domain of a multimeric glycoprotein vWF. vWF is released by Weibel Palade bodies from the endothelial surface and from the alpha-granules of activated platelets, that together, flood to the injured site[12]. Together with thrombin, a locally produced enzyme that is released at the injury site to increase platelet aggregation, more platelets are recruited to the injury site and self-adhere, a process known as aggregation[13]. As will be described in detail next, vWF plays a crucial role in this platelet-

platelet aggregation during the formation of the hemostatic plug. This interaction is summarized in **Figure 1**[14].

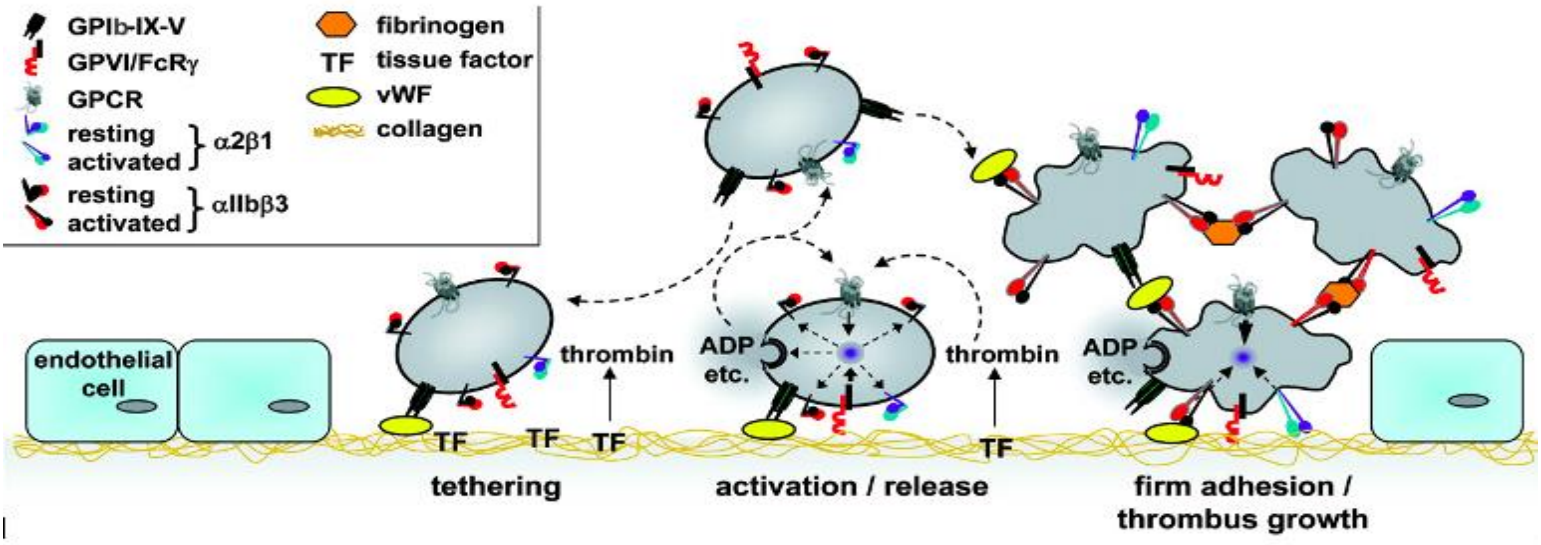


Figure 1: The interaction between Von Willebrand Factor (vWF) and glycoprotein Ib-IX-V (GPIb-IX-V), modified from Varga-Szabo et al.

C. The Role of vWF in Stroke: GPIb-IX-V - vWF Tethering:

As previously defined, vWF is a multimeric glycoprotein involved in the formation of a hemostatic plug (thrombus) when a vascular injury occurs. Studies have demonstrated that incidence of AIS correlates with vWF levels among persons at risk[15,16]. The cascade of interactions that vWF is involved in for a hemostatic plug to form is explained here. **First**, exposed subendothelial ECM collagen at the site of injury interacts with circulating blood platelets via the glycoprotein Ibα (GPIbα) subunit of the GPIb-IX-V complex on the blood platelets surface. vWF serves as a tether between GPIbα and the ECM collagen, and is critical for the initial slowing of flowing platelets[17-20]. Ex vivo studies of human blood have demonstrated that the GPIb-IX-V -

vWF interaction is the primary adhesive interaction in the initiation of blood platelet adhesion with shear rates >1000s^{-1} [21]. These high shear rates are most often found in small arteries, such as the MCA, which make it an attractive target for anti-thrombotic therapies[21]. **Next**, the glycoprotein IIb/IIIa (GPIIb/IIIa) complex is activated due to the initial interaction of GPIb-IX-V with vWF. GPIIb/IIIa activation transitions a "resting state" of integrin $\alpha 2b\beta 3$ to an "activated state" and is characterized by interactions of this integrin with other platelets via both fibrinogen and vWF, allowing for platelet-platelet aggregation/adhesion to occur. **Finally**, the initial interaction of GPIb-IX-V with vWF also activates the glycoprotein Ia/IIa (GPIa/IIa) complex, enabling integrin $\alpha 2\beta 1$ to bind to the exposed collagen of the subendothelial ECM at the injured site as well. **All three** of these major interactions acting in unison are what produce the thrombus, inducing AIS; and all three stem from the initial interaction between GPIbα and vWF **(Figure 1., Figure 2.)**[14,22]. Keeping this process in mind, inhibitors of vWF have been shown to limit thrombosis,[23-26] however, one would expect their effect on hemostasis to lead to significant bleeding in the surgical setting[25-27]. Knowing this, it is clear as to why vWF is so important in the formation of a thrombus, and why a reversible vWF inhibitor would benefit patients requiring platelet inhibition in the perioperative setting.

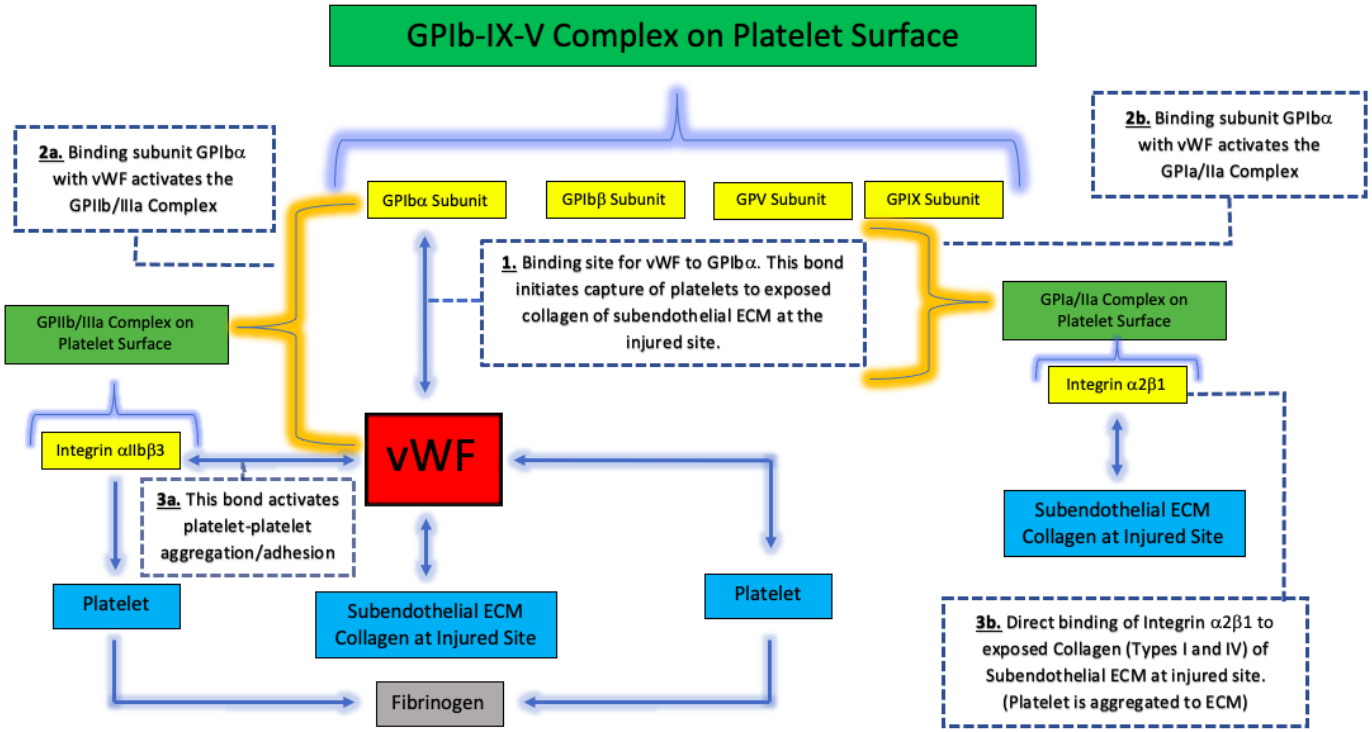


Figure 2. GPIb-IX-V Complex Interaction with Von Willebrand Factor Flow-Chart

D. Aptamers:

Aptamers are RNA or DNA-based oligonucleotide molecules that function by folding into 3-D structures, binding to and inhibiting the function of target proteins with high affinity and specificity[4]. While most antithrombotic therapeutics routinely come with the risk of hemorrhage due to lack of reversibility, antithrombotic aptamers are carefully regulated with a complementary antidote oligonucleotide, showing their potential to safely treat thrombosis. Additionally, Advantages to using aptamers as drug agents include the ability to modify their half-life, unlimited shelf life, low immunogenicity, and because their isolation occurs in vitro, aptamers can be functionally isolated to virtually any protein (REF Nimjee et al. annual Reviews of Medicine 2005).

As previously described, vWF is a promising target for antithrombotic therapeutics as it plays a critical role in activating platelet adhesion, activation and aggregation by its initial binding

to the GPIbα subunit of the GPIb-IX-V complex on the blood platelets surface. Thus, our laboratory has developed an RNA aptamer (DTRI-031) that binds to and inhibits vWF, as well as designed a specific reversal agent (DTRI-025) to regulate its activity. The combination of anti-VWF aptamer DTRI-031 and specific neutralizing oligonucleotide DTRI-025 represents the first rationally-designed drug-antidote pair to treat thrombotic disease.

E. Research Goals:

As stated above, rTPA improves functional and neurological outcomes in AIS patients, but the risk of hemorrhage gives it a small timeframe in which it can be administered (<4.5 hours)[10-11]. Thus, our goal was to develop a more robust treatment for AIS by targeting vWF, the major component of the thrombus seen in patients who suffer AIS (REF: DiMeglio et al. Neurology 2019). By inhibiting vWF, DTRI-031 blocks the initial interaction of the platelet's GPIb-IX-V complex with vWF. This initial inhibition not only prevents platelet adhesion and aggregation in thrombus formation on the exposed ECM of the vessel wall, but can also recanalize a vessel in which an already stable thrombus is present (REF Nimjee et al. Molecular Therapy 2019). Furthermore, by matching DTRI-031 with a specific reversal oligonucleotide DTRI-025, and determining optimum concentration of said antidote, **(Figure 3)** complete reversal of the aptamer can occur, providing a means to prevent devastating intracranial hemorrhaging from occurring and extending treatment time beyond the 4.5 hours that limits rTPA administration. By utilizing a clinically-relevant middle cerebral arterial occlusion (MCAO) stroke model and extending the duration of the stroke to 6 hours before administering treatment, we aimed to demonstrate that DTRI-031 has a safe and efficacious route to thrombolytic outcome beyond the established rTPA protocol.

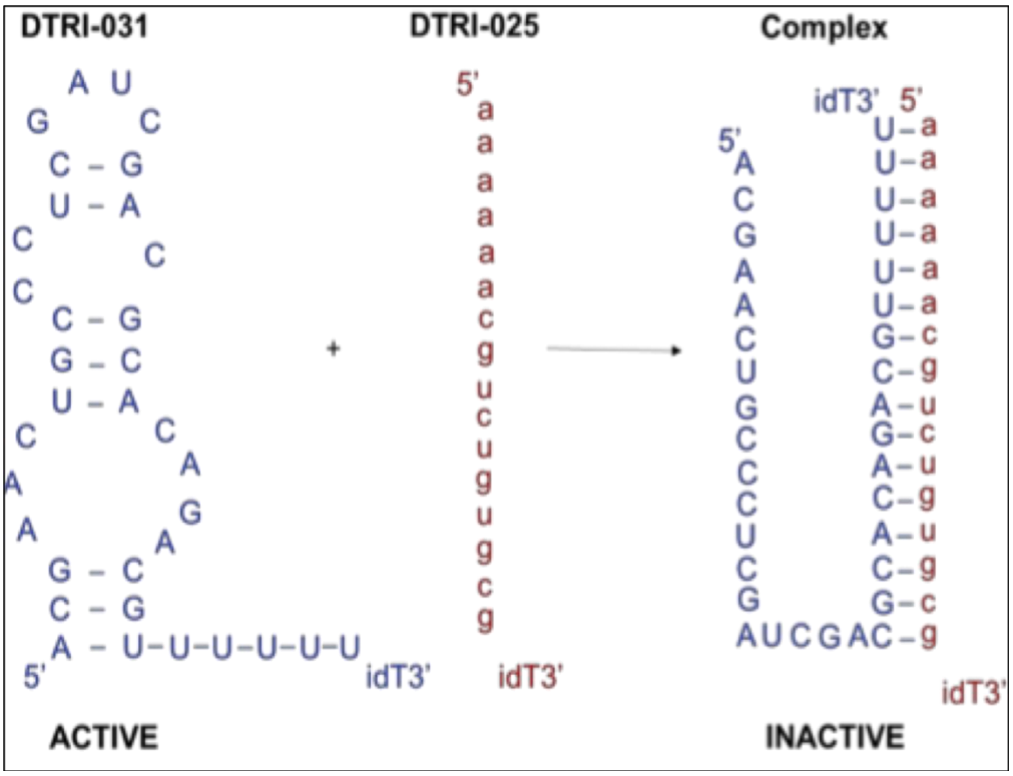


Figure 3: Antidote oligonucleotide (DTRI-025) binds to VWF aptamer (DTRI-031) by Watson Crick base pairing preventing it from binding to the target protein.

III. Materials and Methods:

A. Animals

All experiments were approved by The Ohio State University Institutional Animal Care and Use Committee At The Ohio State University Interventional Cardiology Core, 32 adult hounds purchased from Covance Incorporated (24-45 kg, > 1 year of age) were used to evaluate VWF inhibition by DTRI-031 in a large animal of stroke (Table 1). Hounds (N = 32), were group housed in a temperature and humidity-controlled vivarium at Wiseman Hall and maintained by ULAR staff. Hounds were pre-medicated with intramuscular acepromazine followed by ketamine and midazolam, intubated, placed on a ventilator, and maintained with

continuous inhalation of isoflurane. Deep anesthesia was verified by loss of consciousness, loss of reflex or muscle response, and loss of response to stimuli.

B. Middle Cerebral Arterial Occlusion (MCAO):

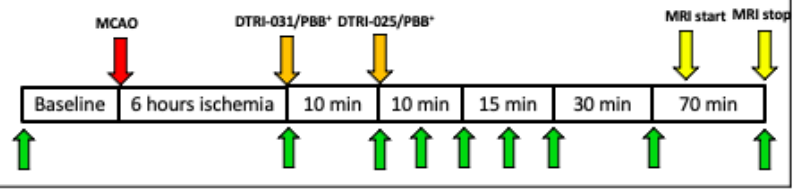
To perform MCAO, animals were positioned supine, with the right femoral artery and vein surgically exposed. A 4-French sheath was placed into the artery for endovascular access, in order to place an occlusive thrombus and perform angiography. A 4-French venous sheath was placed for drug administration, and a second 4-F arterial sheath for timed blood draws. Baseline Platelet Function Analysis (PFA-100), whole blood impedance aggregometry (Chronolog), and buccal bleeding time data were all collected, with materials and methodology having been optimized for canine subjects[29]. An MCA stroke was induced by inserting a micro-catheter into the right MCA artery under fluoroscopic guidance using a GE 9900 Fluoroscope (GE Healthcare, Chicago, IL)[30]. An autologous thrombus was then introduced that had been prepared as described[35,36] and occlusion was verified angiographically. Six hours after MCAO induction, DTRI-031 was administered over 5 minutes at 0.5. 1.0, or 5.0 mg/kg intravenously and whole blood was collected at designated times (**Table 1/Table 2** below). This time point was chosen as it is outside the therapeutic limit for rTPA use. For Aptamer + Antidote experiments, ten minutes after the start of DTRI-031 infusion, DTRI-025 was administered at 0.5 mg/kg to assess DTRI-031 reversibility **(Table 2),** and whole blood was drawn again. DTRI-025 dosing was optimized in previous serial bleed experiments in canine blood. Magnetic resonance imaging (MRI) was performed 7 hours after MCAO. Animals were then sacrificed immediately thereafter, and necropsy was performed to isolate the heart, kidneys, liver, and brain for histopathologic evaluation. All procedures are in

routine use and were performed by the Interventional Cardiology Core, OSUWMC[31]. Canine experiments began October 10, 2020, and continued until April 9, 2021.

Table 1. MCAO Treatment Groups and Animal Numbers

Table 1. MCAO Treatment Groups and Animal Numbers	Aim 1 MCAO
PBB⁺ (Control)	8
DTRI-031 @ 0.5 mg/kg, DTRI-025 @ 0.5 mg/kg	7
DTRI-031 @ 0.5 mg/kg, DTRI-025 @ 5.0 mg/kg	1
DTRI-031 0.5 mg/kg, PBB⁺	8
DTRI-031 1.0 mg/kg, PBB⁺	4
DTRI-031 5.0 mg/kg	4
Total Canines needed to Complete Aim #1 (MCAO and Serial Bleeds will utilize the same hounds)	32

Table 2. MCAO Study Timeline

Table 2. MCAO Treatment Groups and Animal Numbers DTRI-031/DTRI-025 sequential administrations	Hound Numbers
	Hound Stroke MCAO
DTRI-031 0.0 mg/kg (PBB⁺ control), DTRI-025 @ 0.0 mg/kg (PBB⁺ control)	8
DTRI-031 0.5 mg/kg, DTRI-025 @ 0.0 mg/kg (PBB⁺ control)	8
DTRI-031 1.0 mg/kg, DTRI-025 @ 0.0 mg/kg (PBB⁺ control)	4
DTRI-031 5.0 mg/kg, DTRI-025 @ 0.0 mg/kg (PBB⁺ control)	4
DTRI-031 0.5 mg/kg, DTRI-025 @ 0.5mg/kg	7
DTRI-031 0.5 mg/kg, DTRI-025 @ 5.0mg/kg	1
Timeline of experimental protocol = Ischemic Injury = Treatment (5 min bolus) = Magnetic Resonance Imaging (MRI) = Blood draw for PFA, Chronolog, TEG, bleeding time, VWF/ADAMTS13	

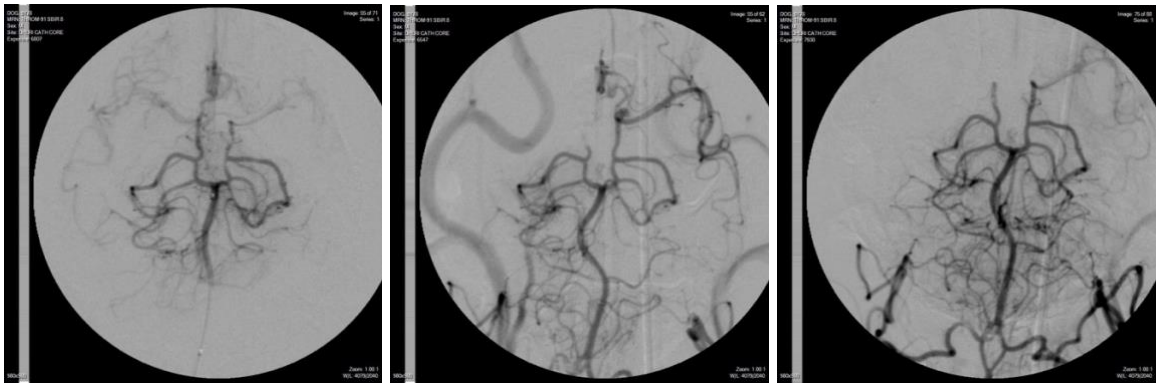
C. In Vivo Visualization of the Induced Thrombus

Referring back to the basic concepts of platelet aggregation and blood clot formation described above and keeping in mind that the definition of AIS involves the blockage of an arterial pathway, cutting off vital nutrients (especially oxygen) to the brain, we made use of an

autologous clot[35,36] via insertion through a 4-French sheath catheter into the Middle Cerebral Artery (MCA) of the Hounds. To verify both MCA occlusion and reperfusion after DTRI-031 and DTRI-025 were given, digital subtraction angiography was performed on the hounds. This was done at baseline, after thrombus placement, and before sacrifice. The angiogram is an invaluable visualization tool as it utilizes a contrast agent at is delivered into the cranial arteries, making them visible on a fluoroscope (GE 9900) x-ray monitor, effectively creating a visual pathway of the cranial arteries **(Figure 4).** To classify percent revascularization of the MCA in hounds, the Thrombolysis in Cerebral Infarction (TICI) grading system was used. The TICI grading system allows for the determination of response to thrombolytic therapy after AIS. More specifically, the TICI method is broken down into four major grades (Grade 0, Grade 1, Grade 2, and Grade 3). Grade 2 also has two subclasses within its domain. All grades and subclasses are defined below in **Table 3.**[37].

Table 3. Definitions for Thrombolysis in Cerebral Infarction (TICI) Method.

Thrombolysis in Cerebral Infarction (TICI) Table	
Grade 0	No perfusion of the treated occluded vessel(s)
Grade 1:	Penetration with Minimal Perfusion
Grade 2A:	Only partial filling (less than two-thirds) of the entire vascular territory is visualized
Grade 2B:	Complete filling of all the expected vascular territory is visualized but filling is slower than when at hemostasis
Grade 3:	Complete perfusion of the treated occluded vessel(s)



A. B. C.

Figure 4. Use of fluoroscopic angiography allowed for verification of baseline MCA arterial flow (A.), followed by MCAO (B.), and finally reperfusion at sacrifice after DTRI-031infusion (C.)

D. Quantification of Infarct Volume

Brain MRI was performed immediately before sacrifice utilizing a Siemens Prisma 3 Tesla MRI scanner (Siemens, Munich Germany). An ECG-gated breath-hold pulse sequence requiring a 10-15 second hold was used, depending on the animal's heart rate. MRI capture protocol included single slice horizontal long axis (4 chamber view) steady-state free precision cine acquisition (8 mm slice thickness), multi-slice short axis cine acquisitions (8mm slice thickness, 0 mm separation), multi-slice short axis double inversion recovery fast spin echo imaging) and bright blood acquisitions (using steady state free precession imaging). For stroke-volume calculation from 2D images, we converted raw MRI images to DICOM format, importing them into Image J software. Most importantly, by delineating both whole brain and injured brain borders, we were able to quantify whole brain and infarct areas **(Figure 5).** Infarct regions were determined by outlining the affected region and verified in TTC staining (explained

next). Hound MRI was performed at The Ohio State University Center for Cognitive and Behavioral Brain Imaging (CCBBI).

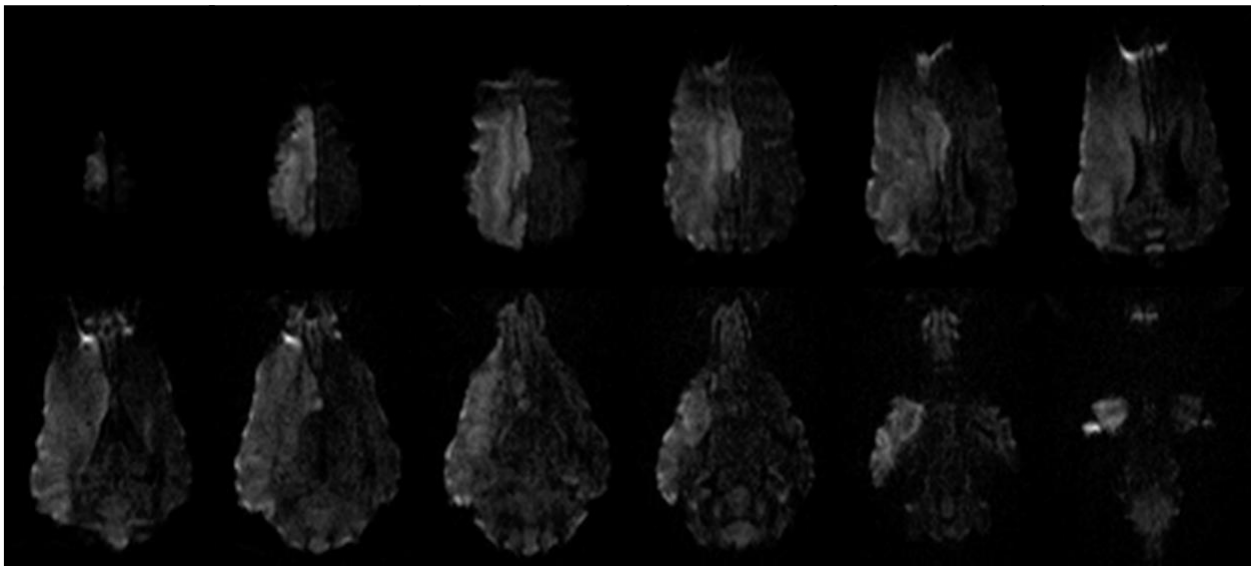


Figure 5. T2 Weighted MRI imaging allowed for quantification of infarct volumes following MCAO

E. TTC Verification of Infarct Volumes and Reperfusion of Vessels

We verified infarction and hemorrhage volumes, found on MRI, on a macro scale by obtaining consecutive coronal sections of hound brain and staining with triphenyl-2,3,5-tetrazolium-chloride (TTC) to differentiate between metabolically active (red) and inactive (white) cells **(Figure 6).** TTC staining was performed by the Pathology Core at The Ohio State University College of Veterinary Medicine, contracted by Matthew Joseph at the OSUWMC ICC.

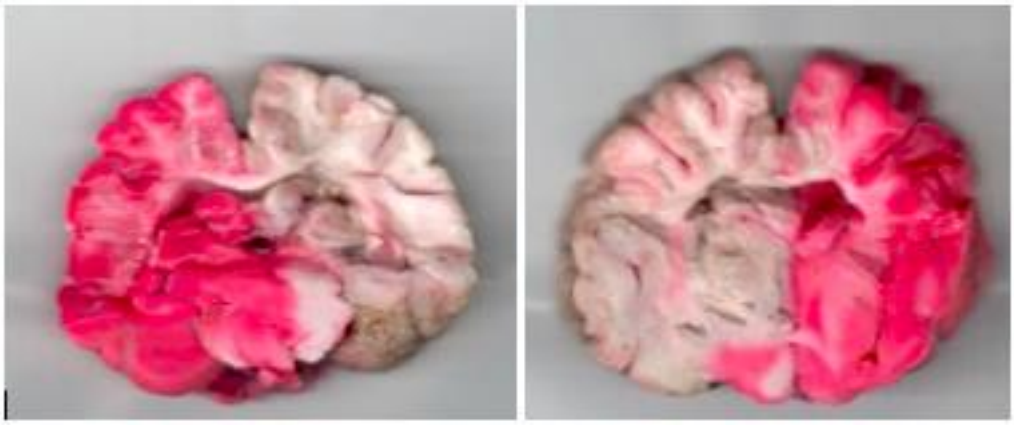


Figure 6. TTC stained (front and back) coronal brain section of hound (red is alive, white is dead)

F. Platelet Reactivity Tests: Platelet Function Analysis (PFA), Chrono-log Aggregometry, and Buccal Mucosa Bleeding Time

Verifying platelet reactivity as a result of DTRI-031 administration and DTRI-025 neutralization was evaluated to ensure the safety and efficacy of the aptamer. To do this, platelet function analysis (PFA) was utilized to determine how well the subjects' platelets were aggregating. The platelet function analyzer 100 (PFA-100) is a bench-top automated point-of-care instrument that assesses primary hemostasis under shear stress. The PFA-100 uses a disposable test cartridge that contains a membrane impregnated with collagen plus ADP (Col/ADP membrane) or epinephrine (Col/Epi membrane)[38]. Whole Blood Impedance Aggregometry (WBIA) (Chrono-log) was used to verify how well the subjects' platelets were aggregating once subjected to administered agonists. WBIA measures the change in electrical impedance between two electrodes when platelet aggregation is induced by an agonist. The method is performed using whole blood and so eliminates the need to generate platelet rich plasma [PRP][39]. Optimal concentrations of agonists were used and included Collagen, ADP, and

Botrocetin. Lastly, Buccal Mucosa Bleeding Time (BMBT) was collected to determine the time required for clotting on the endothelium of the site of injury to occur in vivo. BMBT was performed by activating the Accriva Diagnostics Surgicutt™ Bleeding Time Device (Accriva Diagnostics SU50I)[40] at dimensions of 0.1 cm depth x 0.5 cm length on the endothelium of the animal, and timing the resulting bleeding until clotting had finished. All three of these platelet function analyses are based on the basic concepts we described in our introduction; that injury to the subendothelial ECM of the vessel triggers the initial activation of the GPIb-IX-V complex's GPIbα subunit with vWF, which results in a cascade of clotting events, leading to thrombus formation and subsequent AIS.

G. Data Analysis

Statistical analysis of collected data was performed via the software programs GraphPad Prism and Excel. and expressed as mean ± standard deviations. For comparison between two groups, significance will be determined by paired or unpaired Student *t* test. For comparison of multiple groups, multifactorial ANOVA with post hoc comparison will determine significance. For all data analysis, probability values of $p < 0.05$ were considered significant. See **Table 1** for all experimental outcomes.

IV. Results

A. In Preliminary Data DTRI-031 Administration demonstrated overall reduced infarct volume

After thrombus stabilization was verified, a 6-hour timer was started to allow the stroke to develop, after which time DTRI-031 was administrated. Scheduled angiography and blood draws were performed 10 minutes and 1 hour after DTRI-031 administration. The animals were then transported for brain MRI utilizing a Siemens Prisma 3 Tesla MRI scanner (Siemens, Munich Germany). As stated above, an ECG-gated breath-hold pulse sequence requiring a 10-15 second hold was used, depending on the animal's heart rate. MRI of hounds treated with negative (Platelet Binding Buffer (PBB^{+})) control 6 hours after MCAO developed an infarct 47.0 +/- 6.7% (N = 7). Overall, animals treated with DTRI-031 developed an infarct 44.2 +/- 15.5% (N = 8). Animals treated with DTRI-031 resulted in revascularization in 5 of 8 hounds (62.5%). The infarct volumes of animals that underwent DTRI-031-mediated revascularization was 29.3 +/- 9,4%, which was significantly less than the negative control group ($p<0.5$, utilizing an unpaired t-test) (**Figure 7**.

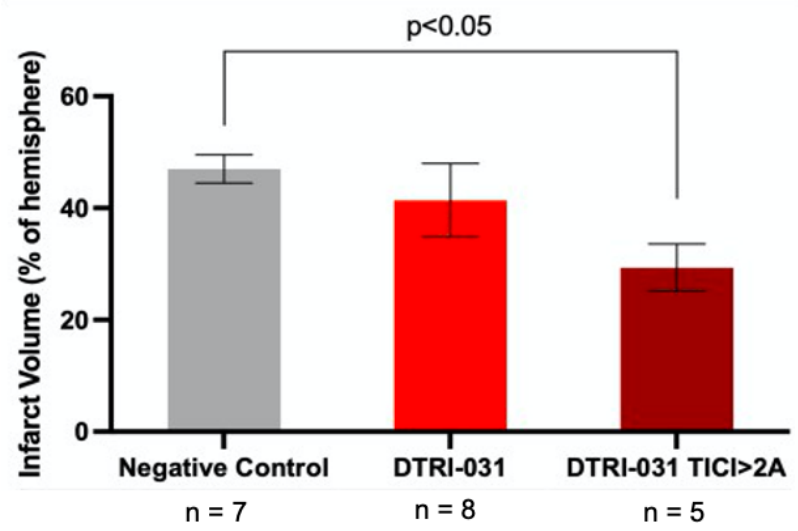


Figure 7. DTRI-031-mediated recanalization reduces infarct volume

B. DTRI-031 Administration inhibited platelet reactivity

In addition to reduced infarct volume, administration of DTRI-031 demonstrated inhibition of platelet reactivity. BMBT, PFA, WBIA were the three parameters we used for testing platelet reactivity to DTRI-031 and DTRI-025. For BMBT, our results indicated that DTRI-031 at concentrations of 5.0mg/kg and 1.0 mg/kg demonstrated the most efficacious platelet inhibition times. Specifically, 1.0 mg/kg showed a longer overall clotting time, but 5.0 mg/kg held clotting time more steadily for a longer period of time. When DTRI-025 was administered after DTRI-031, durable aptamer reversal was witnessed with clotting times returning to normal in as little as 10 minutes (**Figure 8)**. PFA showed similar results with DTRI-031 concentrations of 5.0 mg/kg and 1.0 mg/kg presenting the longest closure time in response to aggregation. Again, 5.0 mg/kg held a steady closure time for the longest period of all tested concentrations (300 seconds for approximately 175 minutes) before inhibition began to diminish. Similarly, DTRI-025 quickly and durably reversed aptamer inhibition in as little as 10 minutes (**Figure 9**). Lastly, WBIA had similar results to both BMBT and PFA with DTRI-031 concentrations of 5.0 mg/kg and 1.0 mg/kg showing the lowest overall impedance values, indicating the greatest inhibition of platelet reactivity. Just as was seen for DTRI-025 in the other two testing parameters, durable reversal of the aptamer occurred with impedance values returning to normal in as little as 10 minutes, indicating reversal of platelet inhibition (**Figure 10).** (all other WBIA graphs can be found in the appendix). Note, all graphs start at 400 minutes as this was the time in which testing began relative to the experiment start time. Agreement among all three platelet reactivity testing parameters demonstrates consistency in the ability of DTRI-031 to inhibit platelet reactivity and in DTRI-025 to rapidly, and durably, reverse that inhibition.

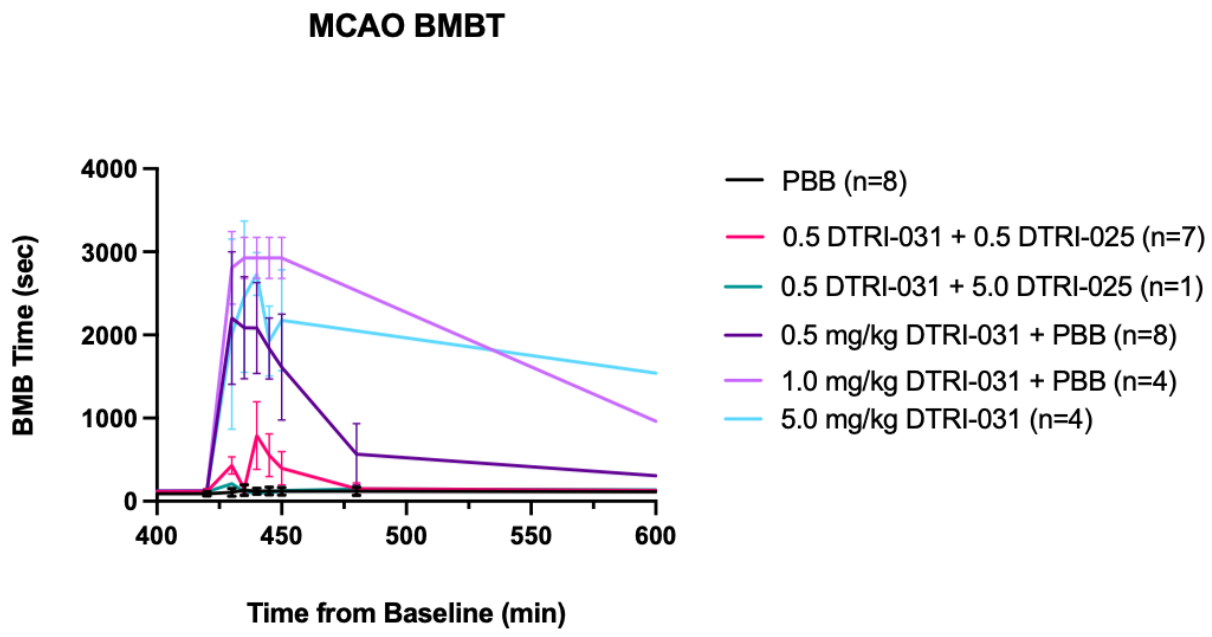


Figure 8. MCAO Hound Buccal Mucosa Bleeding Time

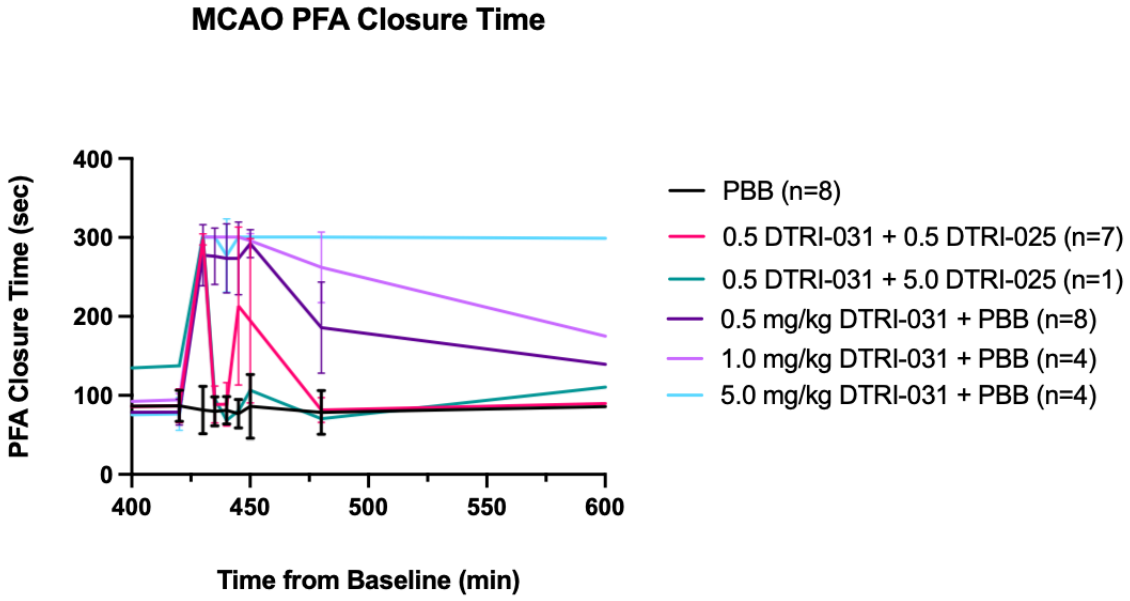


Figure 9. MCAO Hound PFA Closure Time

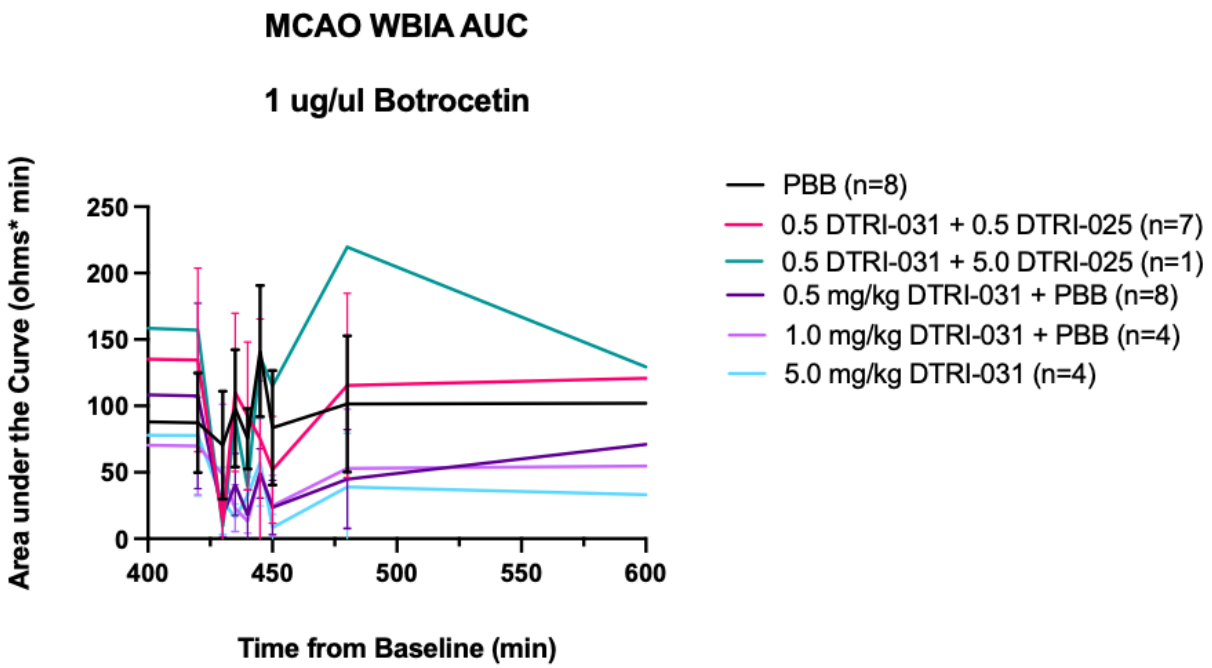


Figure 10. MCAO Hound Whole Blood Impedance Aggregometry Area-Under-the-Curve (AUC) for 1ug/ul Botrocetin Agonist Addition

C. DTRI-031 Resulted in Increased Reperfusion of the MCA Compared to Control

DTRI-031 recanalized the hound MCA after 6 hours of LVO stroke in 5 out of 8 dogs (62.5%), compared to no reperfusion in PBB^{+} (Saline) control **(Figure 11).** Digital subtraction angiography (DSA) was taken 1 hour after treatment and demonstrated no revascularization (a TICI grade of 0) in control (N = 7) as compared to DTRI-031 treatment, which resulted in a TICI grade of 2A in 12.5%, 2B in 37.5%, and all the way to a grade 3 in 12.5% of cases (N = 8). These results demonstrate the ability of DTRI-031 to effectively lyse thrombi in vivo.

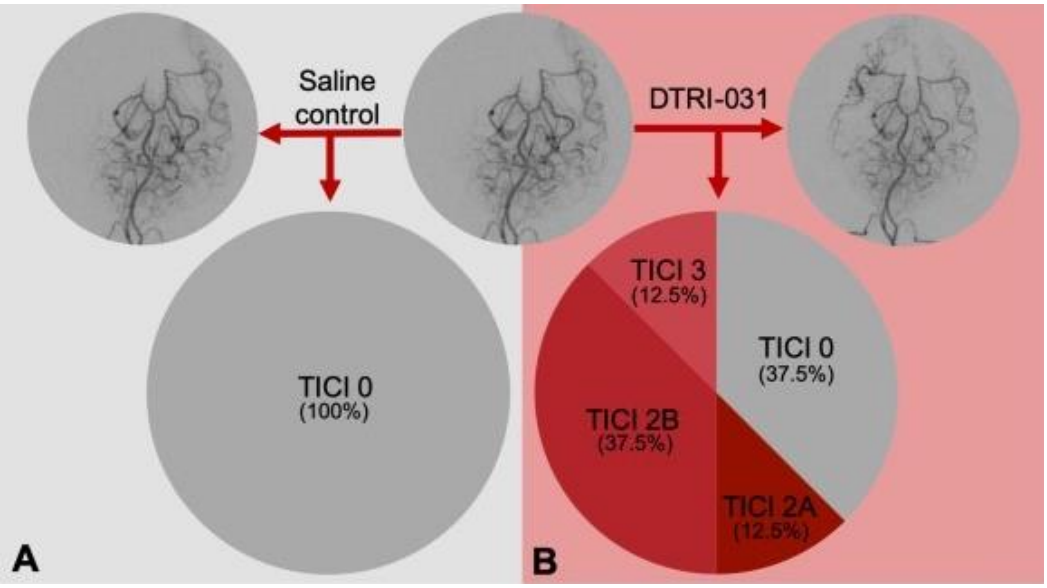


Figure 11. TICI-Based Reperfusion Percentages of MCAO Hounds

D. DTRI-031/DTRI-025 Administration did not alter Canine Physiological Parameters:

Administration of DTRI-031, and its subsequent neutralization with administration of DTRI-025) demonstrated no negative effects on animal physiology. Vital signs (Heart Rate, Mean Arterial Pressure, Mean Systolic Pressure, and Mean Diastolic pressure) were recorded throughout the entirety of the experiment, in order to ensure normal physiological conditions were maintained within the animal. Additionally, White Blood Cell counts and Platelet Counts (WBC, PC) were taken at baseline and before sacrifice, also to ensure normal physiological conditions were maintained within the animal. No significant difference was found in the mean blood pressures or heart rates between treatment groups. Normal increase in WBC count was seen and overall PC was maintained for all groups (DTRI-031 5.0mg/kg did not undergo WBC/PC counts due to logistical issues). All treatment groups resulted in normal physiological conditions during the entire surgical procedure (**Figures 12.-17).**

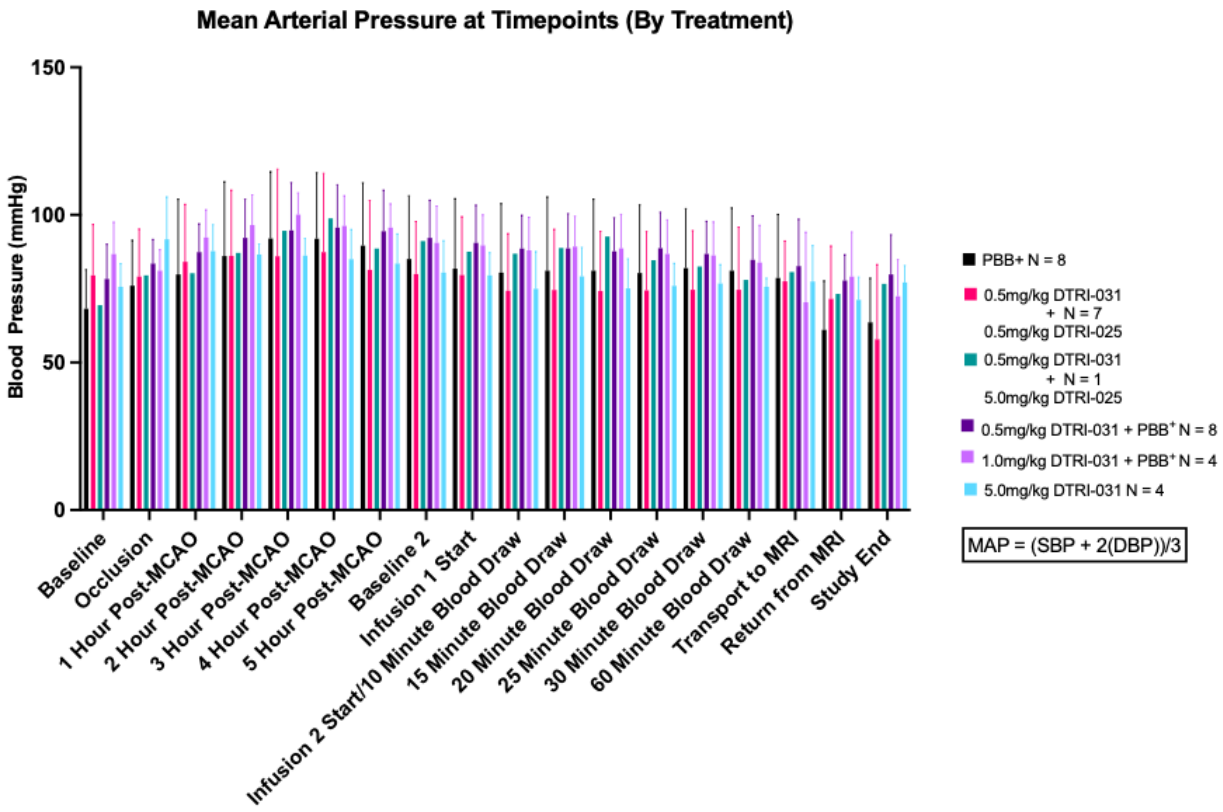


Figure 12. Mean Arterial Pressure at Timepoints (By Treatment) in MCAO Hounds

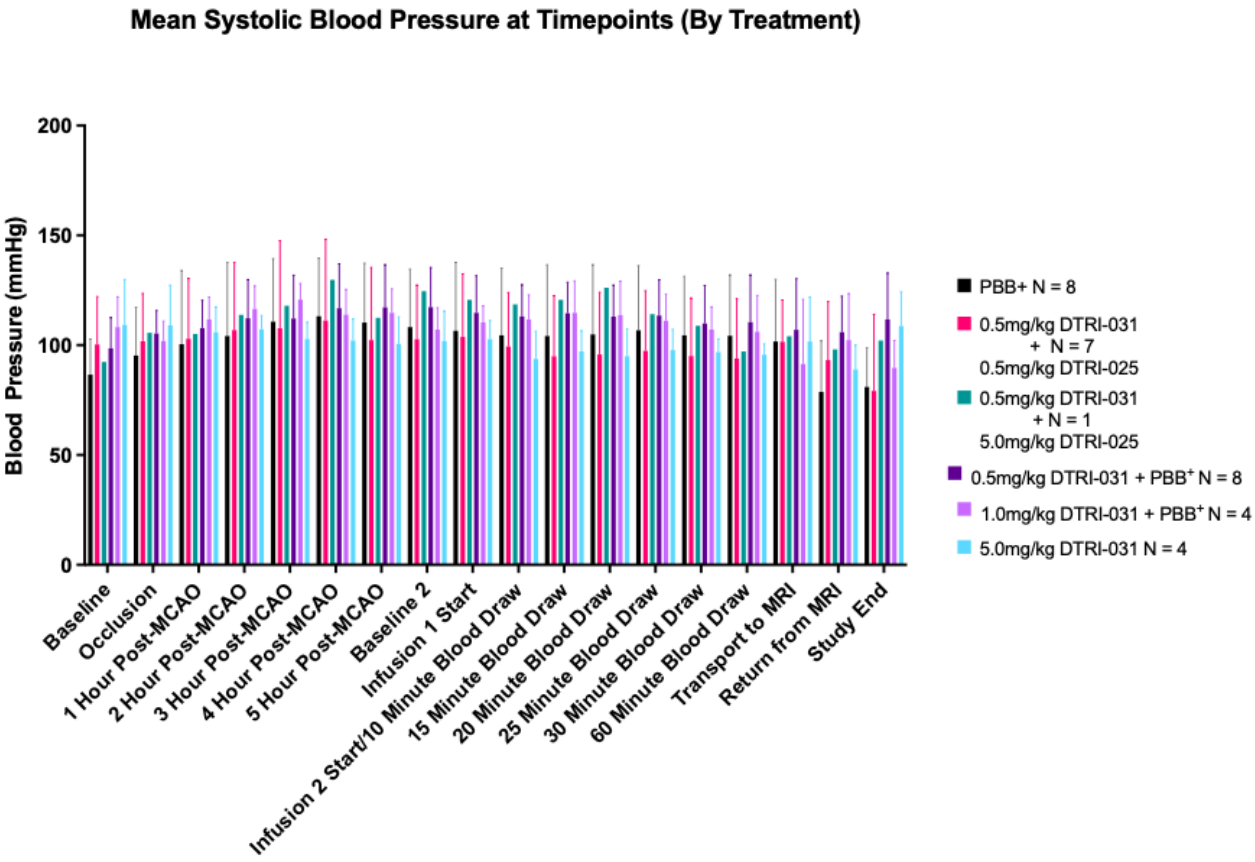


Figure 13. Mean Systolic Blood Pressure at Timepoints (By Treatment) in MCAO Hounds

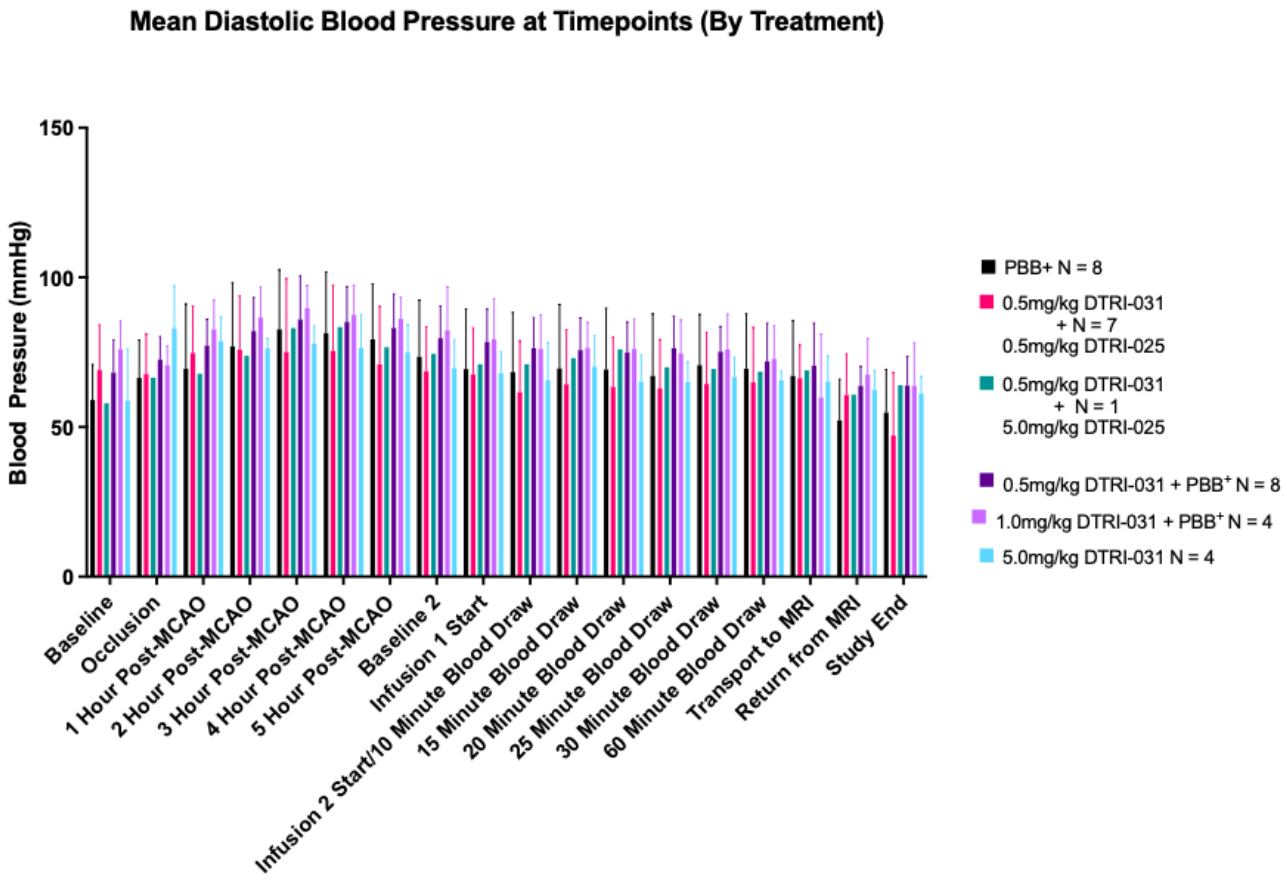


Figure 14. Mean Diastolic Blood Pressure at Timepoints (By Treatment) in MCAO Hounds

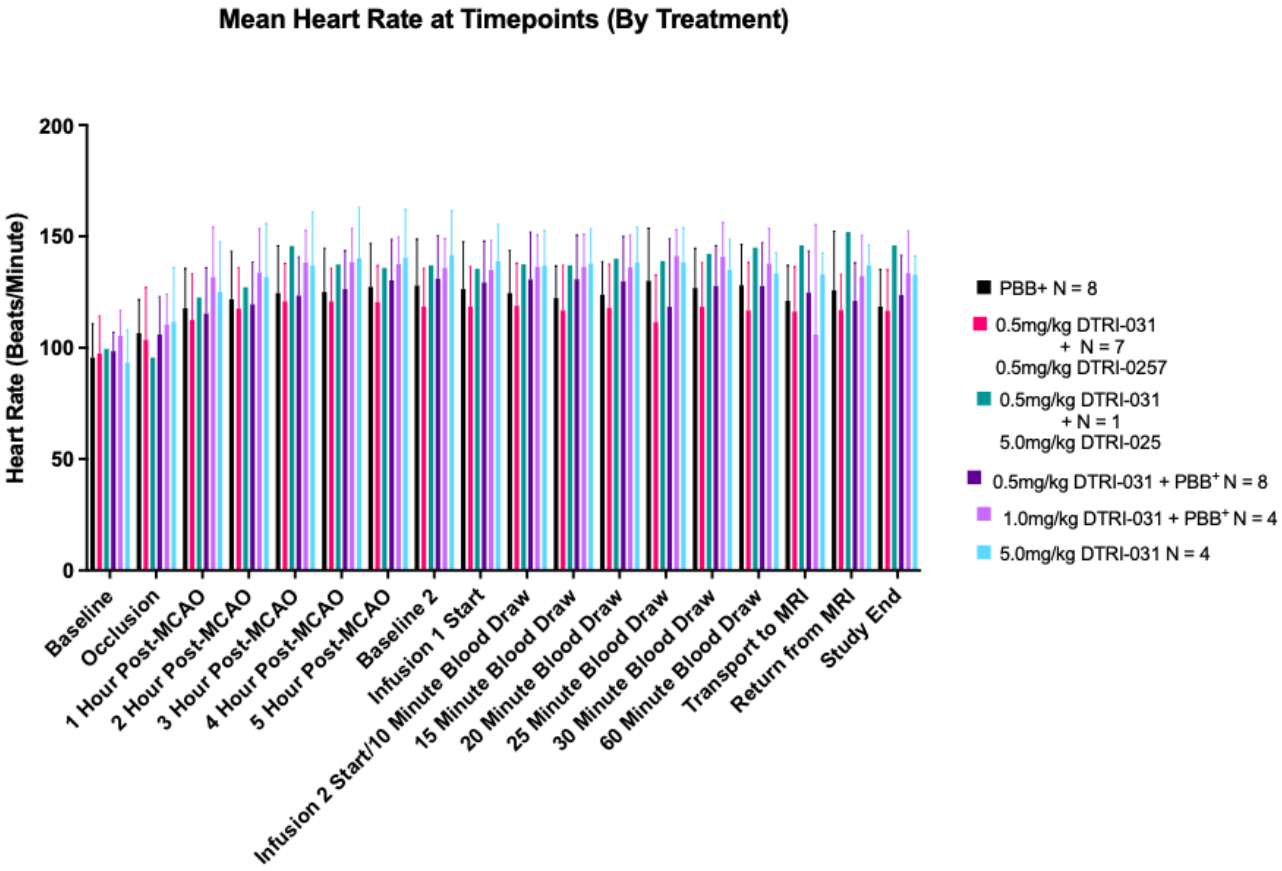


Figure 15. Mean Heart Rate at Timepoints (By Treatment) in MCAO Hounds

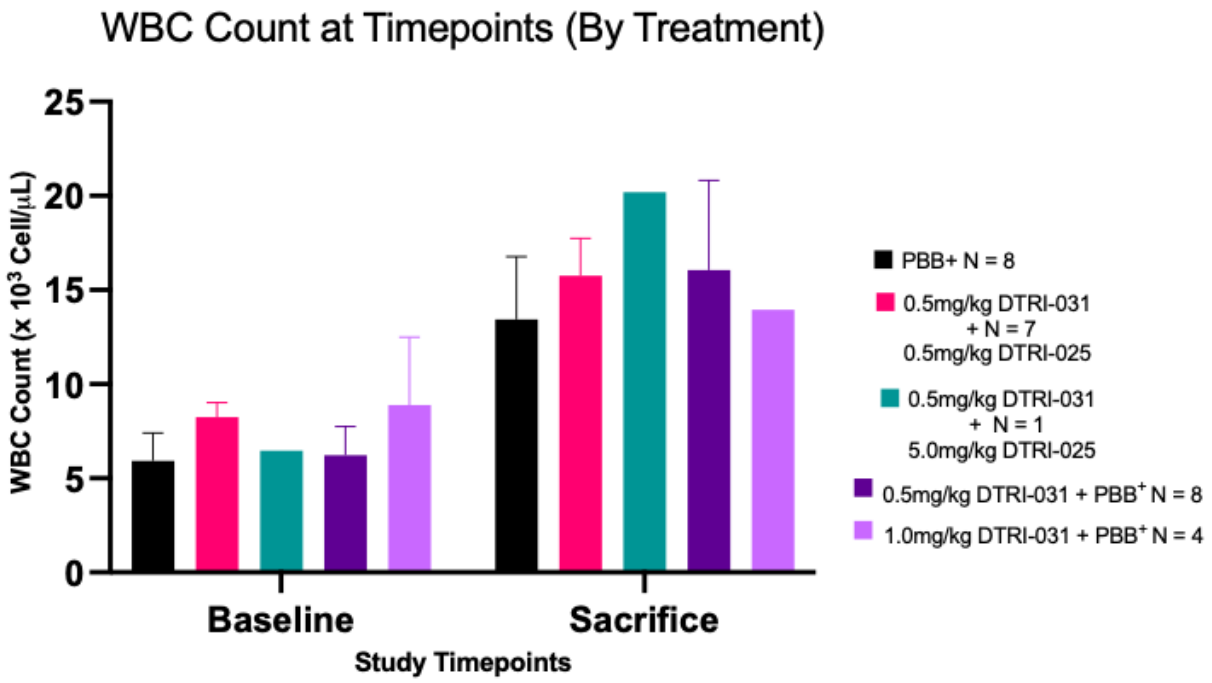


Figure 16. WBC Count at Timepoints (By Treatment) for MCAO Hounds

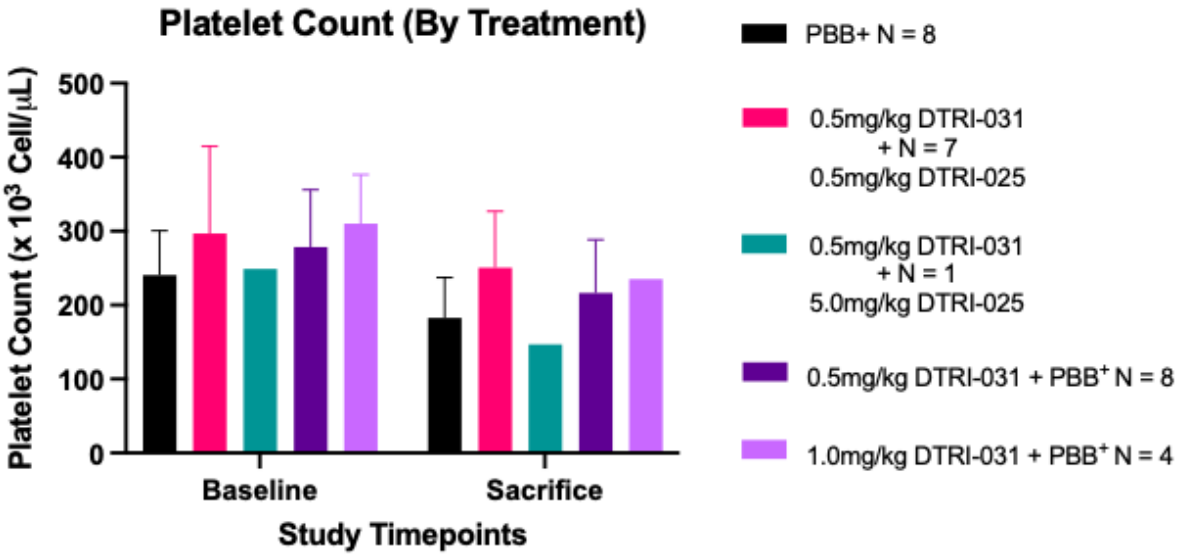


Figure 17. Platelet Count at Timepoints (By Treatment) for MCAO Hounds

V. Discussion

Acute Ischemic Stroke is at the forefront of translational medicine research because of the high prevalence, morbidity and mortality of the disease. Furthermore, over 85% of strokes are ischemic, and over half of all ischemic strokes originate in the Middle Cerebral Artery (MCA)[1-2,6]. Currently, standard stroke treatment includes intravenous infusion of recombinant tissue plasminogen activator (rTPA), a serine protease that's primary function is to eliminate occlusion caused by blood clots via thrombolysis[3]. This method is only marginally effective at thrombolysis, but also cannot be reversed, and must be given within a timeframe of 3-4.5 hours of stroke onset, due to increased risk of hemorrhage into the brain. Potential replacement stroke therapies have been researched in the pre-clinical setting, but only a few have been effective when applied to the clinical setting[33]. von Willebrand Factor (vWF), a known glycoprotein mediator in platelet adhesion and aggregation, plays a major role in vessel injury hemostasis, is an optimal target for inhibition to treat

As was described in the introduction, vWF is a main contributor to both platelet activation and aggregation at the site of injury: First, exposed subendothelial ECM collagen at the site of injury interacts with circulating blood platelets with vWF serving as a tether between the GPIb-IX-V complex on the platelet and the ECM collagen. This step is critical for the initial slowing of flowing platelets.[17-20] Next, the GPIIb/IIIa complex on the platelet is activated due to the initial interaction of GPIb-IX-V with vWF, allowing for platelet-platelet aggregation/adhesion to occur. Finally, the initial interaction of GPIb-IX-V with vWF also activates the GPIa/IIa complex, enabling further platelet binding to the exposed collagen at the injured site as well. All three of these major interactions acting in unison are what produce the

thrombus, inducing AIS; and all three stem from the initial interaction between the platelet and vWF **(Figures 1 and 2)**[14,22].

In past studies, DTRI-031 has shown efficacy in its ability to inhibit VWF and perform in vivo as an anti-platelet agent, preventing thrombus formation[34]. In this study we aimed to investigate the thrombolytic efficacy of DTRI-031. Utilizing clinically relevant stroke models with hounds, Acute Ischemic Stroke was demonstrated by occluding the middle cerebral artery of hounds and allowing a stroke to propagate for 6 hours after occlusion. The thrombolytic efficacy of DTRI-031 was then tested and compared to (PBB^+) (saline) control. Our findings showed that DTRI-031 resulted in smaller infarct size than PBB^+ control **(Figure 7)** with 0.5 mg/kg, which in turn, resulted in the smallest infarct size and greater overall reperfusion **(Figure 11).** Additionally, DTRI-031 administration resulted in longer platelet clotting times in vivo **(Figure 8),** longer time for blood flow to stop when aggravated ex vivo (reported as closure times) **(Figure 9),** and lower overall impedance when platelets were subjected to agonists ex vivo, suggesting less platelet-platelet activation **(Figure 10).** All three of these platelet activity testing parameters showed inhibition of the basic GPIb-IX-V platelet complex-vWF interactions that were described in the introduction. This inhibition is what prevents the cascade of clotting events that result in thrombus formation and AIS. Finally, no adverse effects to hound baseline physiology were found **(Figures 12-17).**

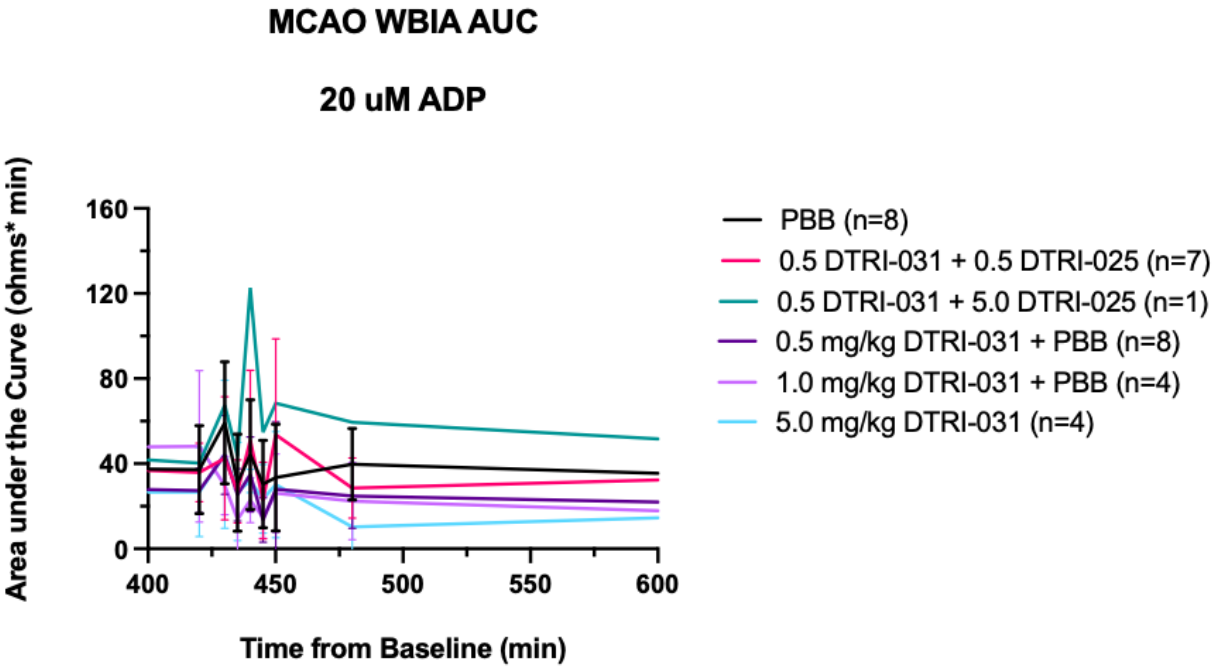
The results presented in this study demonstrate the ability of DTRI-031 to inhibit vWF effectively, and highlight its role as a thrombolytic agent in a large animal model of thromboembolic stroke. This is an ongoing study, and our team believes that with further investigation, DTRI-031 inhibition of vWF can provide a treatment option other than rTPA for those 91.5% of stroke patients who cannot receive rTPA and are left with little options for acute

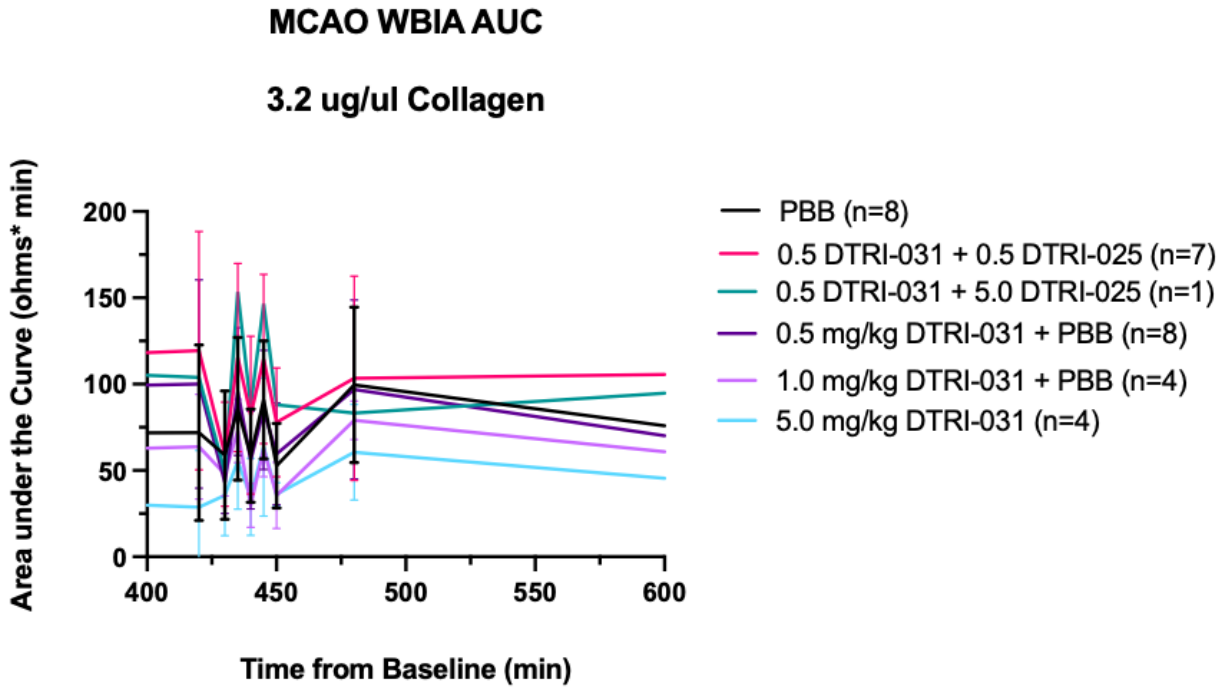
therapy. Furthermore, we believe that pairing DTRI-031 with its complementary reversal agent, DTRI-025, will provide a measure of defense to mitigate the hemorrhaging side effects that currently plague stroke treatment. Potential future applications could be administration of the DTRI-031 in veno-occlusive disease such as deep vein thrombosis and pulmonary embolus, as well as possibly loading the drug on a stent, which could be used as a preventative measure in potential at-risk stroke patients.

VI. Acknowledgements

I would like to thank my laboratory PI Dr. Shahid Nimjee, and laboratory manager, Mrs. Debra Wheeler. Their continued support and guidance for me over the past four years has been a large part of where I am today, and where I am going. I would also like to thank my peers in Dr. Nimjees lab for their collaboration with my experiments, and their friendship. Without them I would not have been able to complete this thesis. They have made my lab experience an enjoyable one and I look forward to seeing where they go in their futures. Lastly, I would like to thank my family and friends for their continued support in my pursuit of medicine as a career, and for being there for me when I needed them.

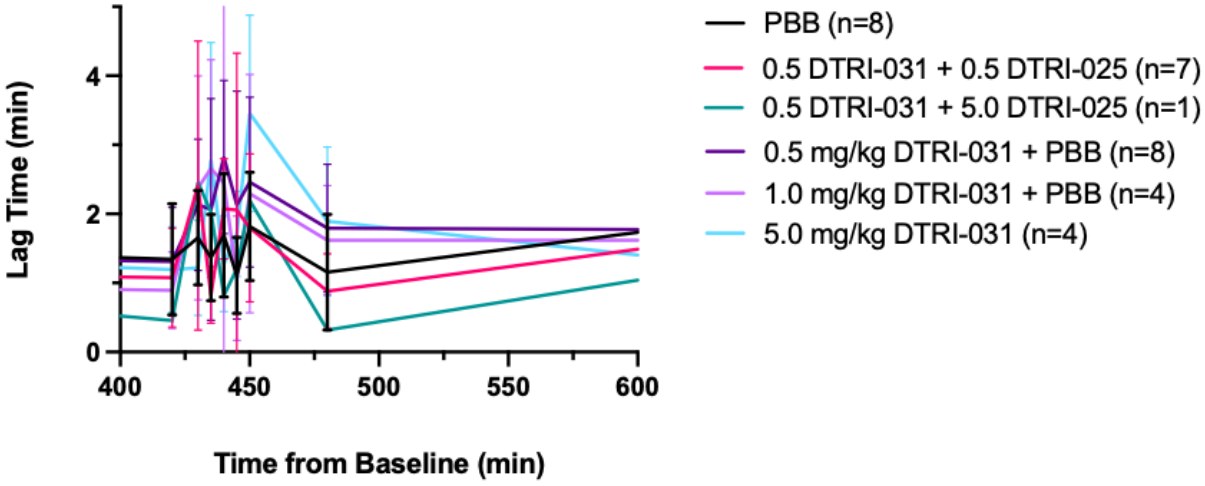
VII. Appendix: Whole Blood Impedance Aggregometry Figures





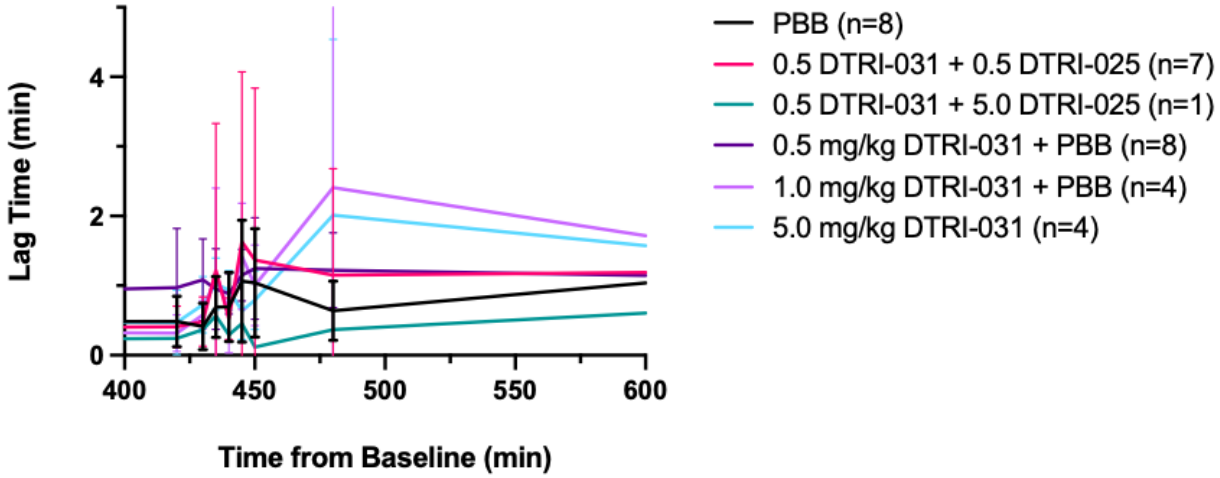
MCAO WBIA Lag Time

1 ug/ul Botrocetin



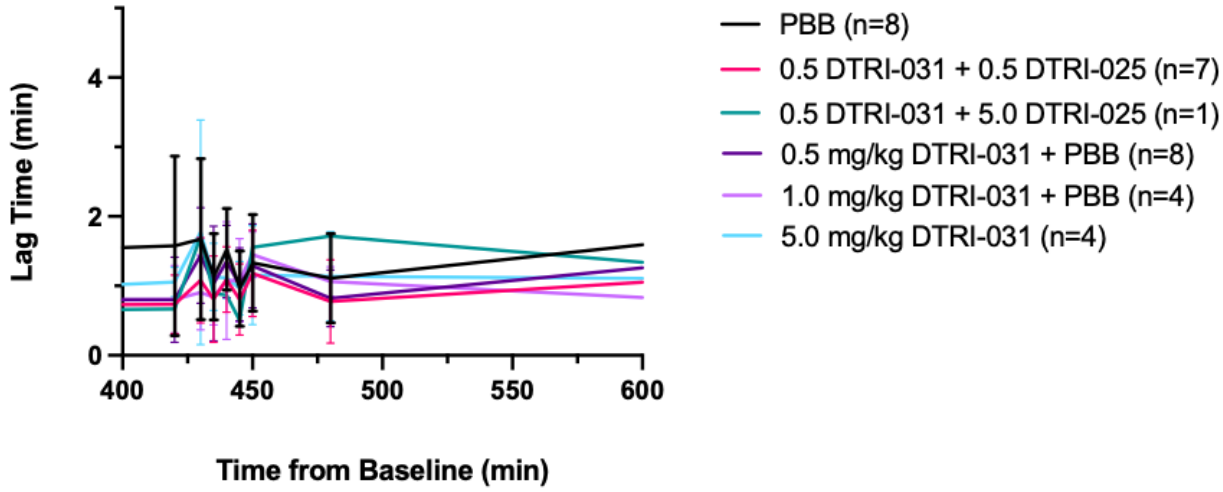
MCAO WBIA Lag Time

20 uM ADP



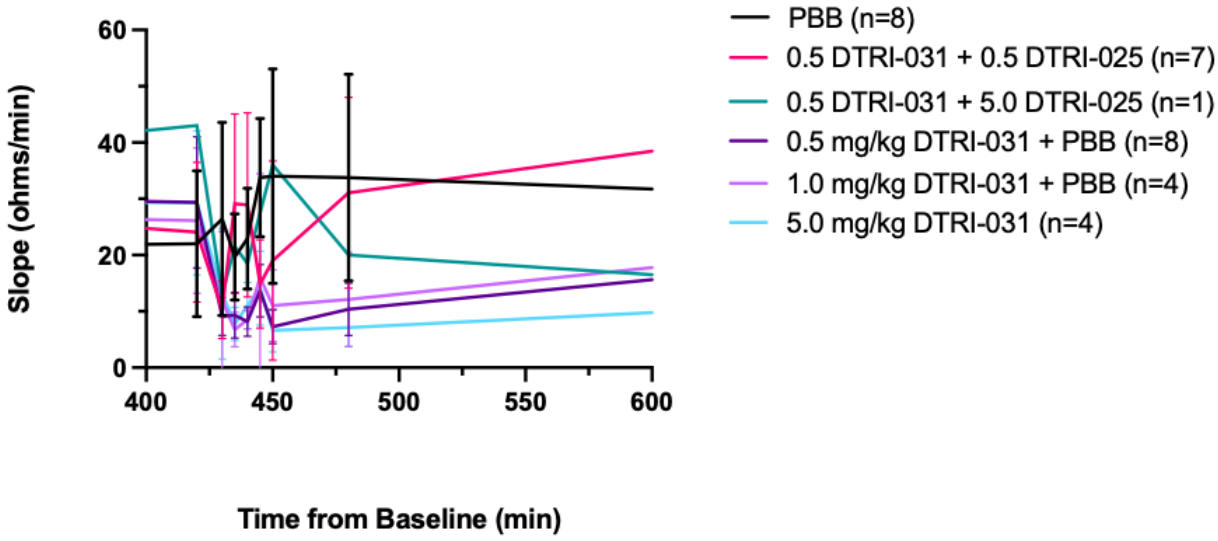
MCAO WBIA Lag Time

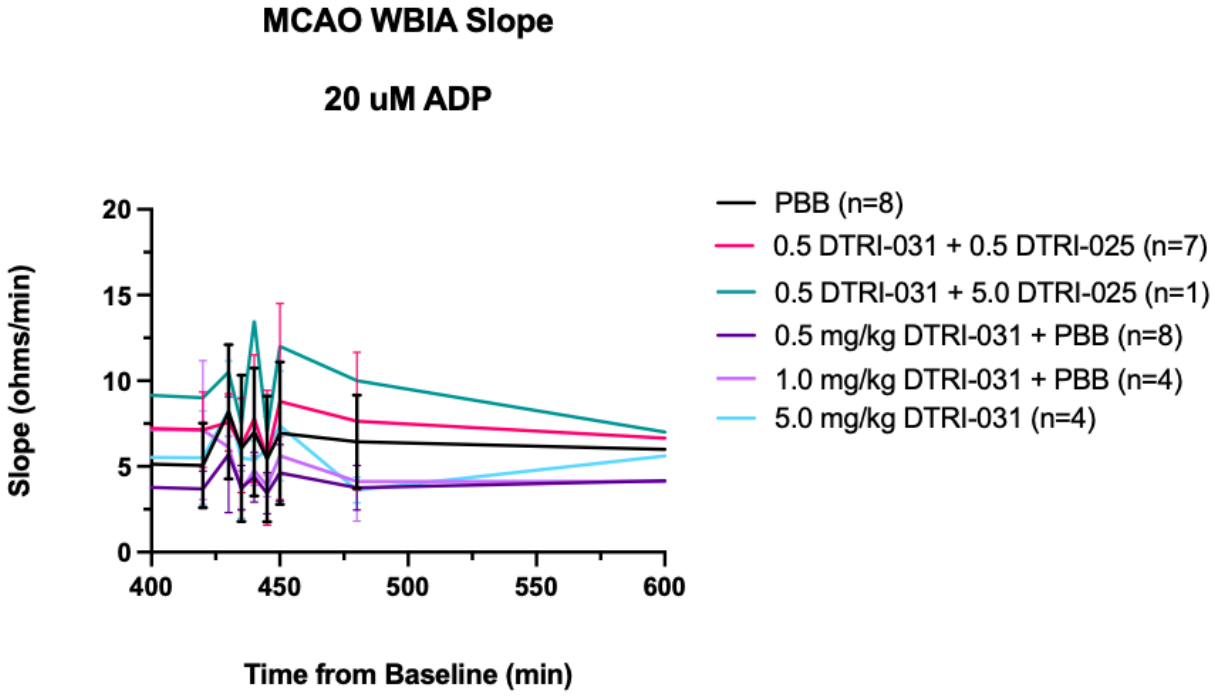
3.2 ug/ul Collagen

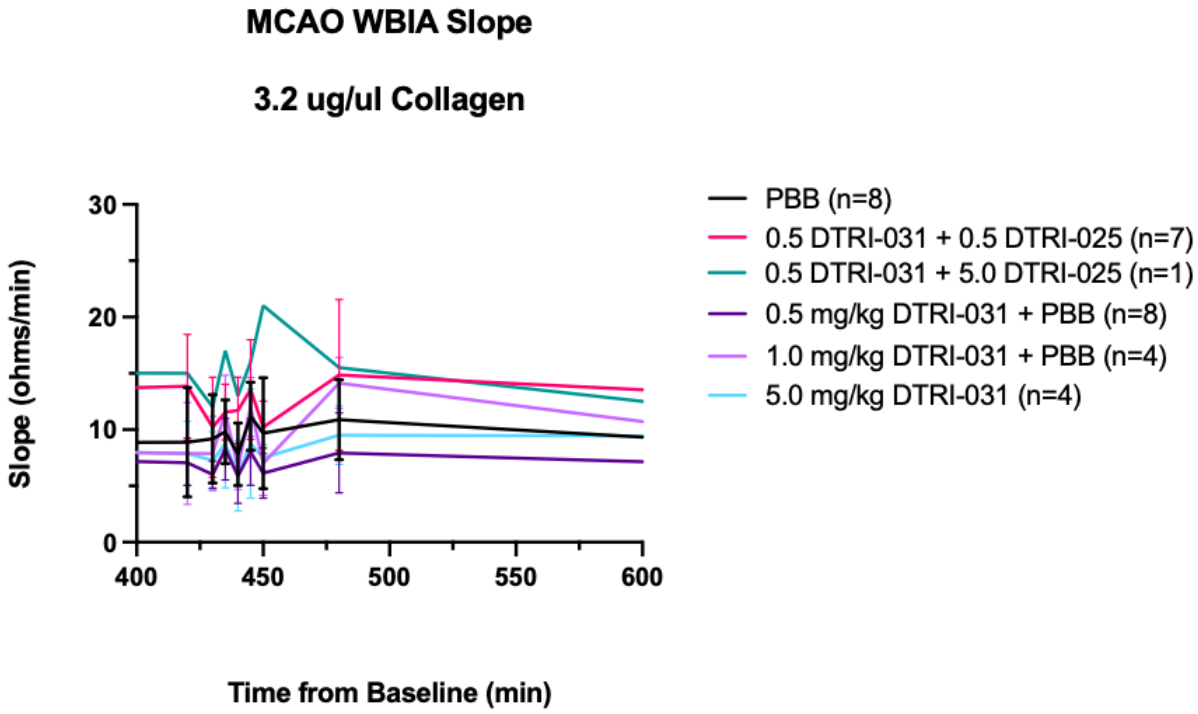


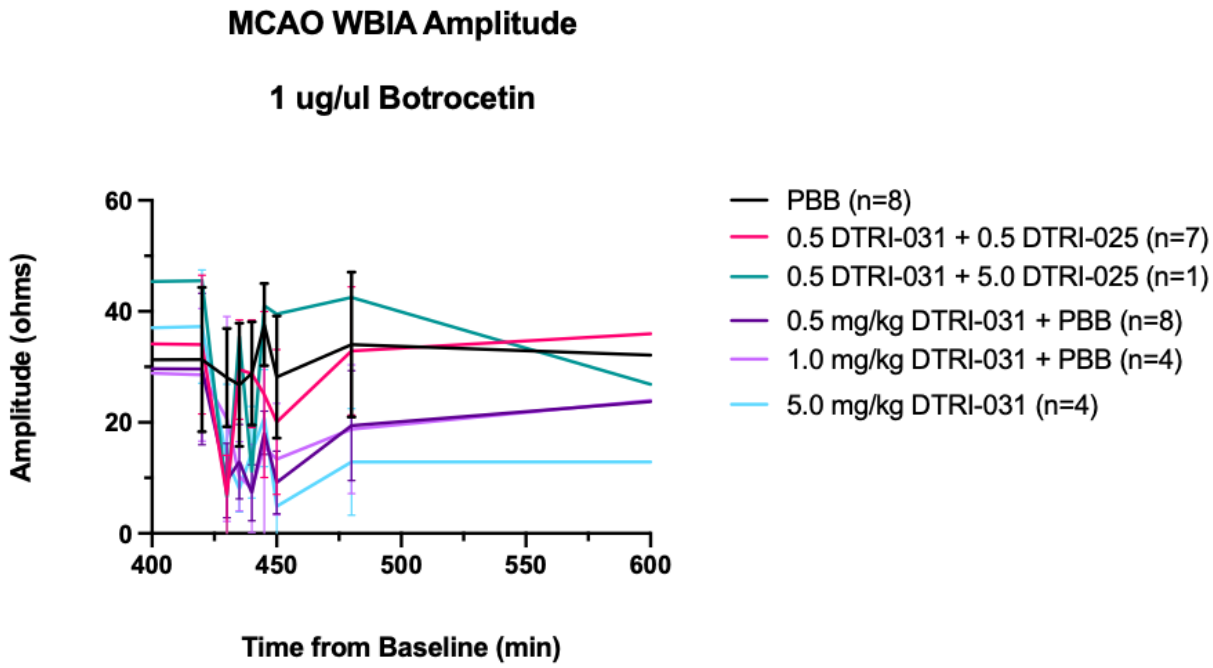
MCAO WBIA Slope

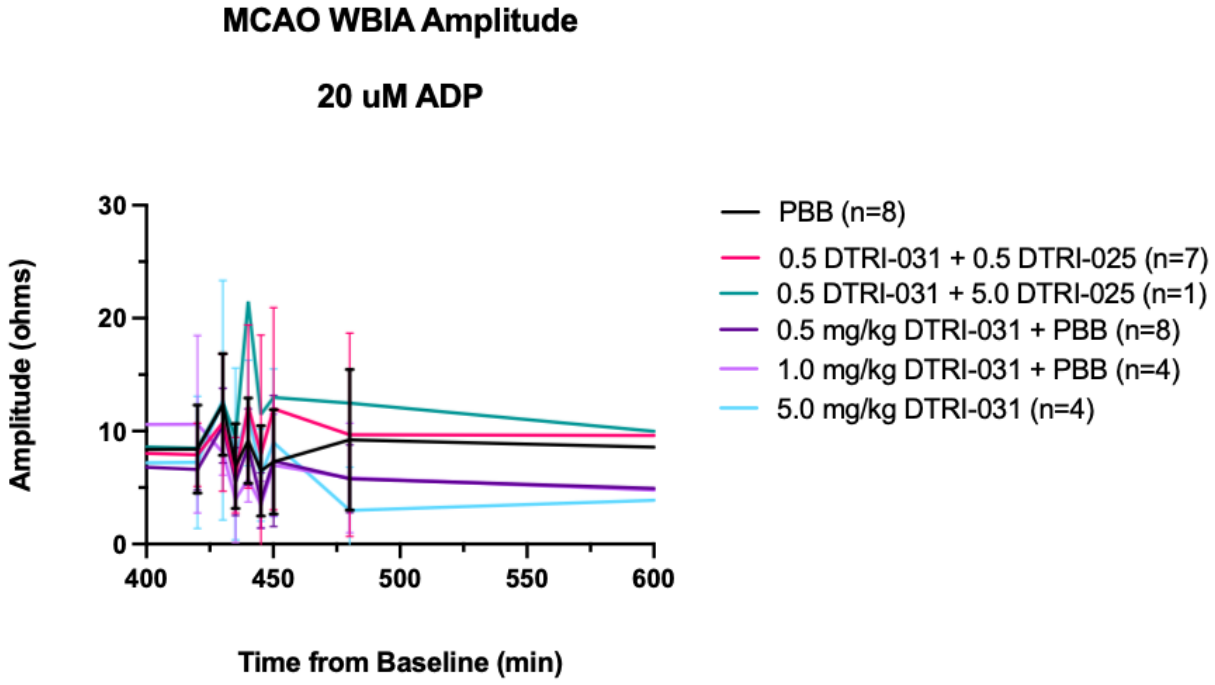
1 ug/ul Botrocetin

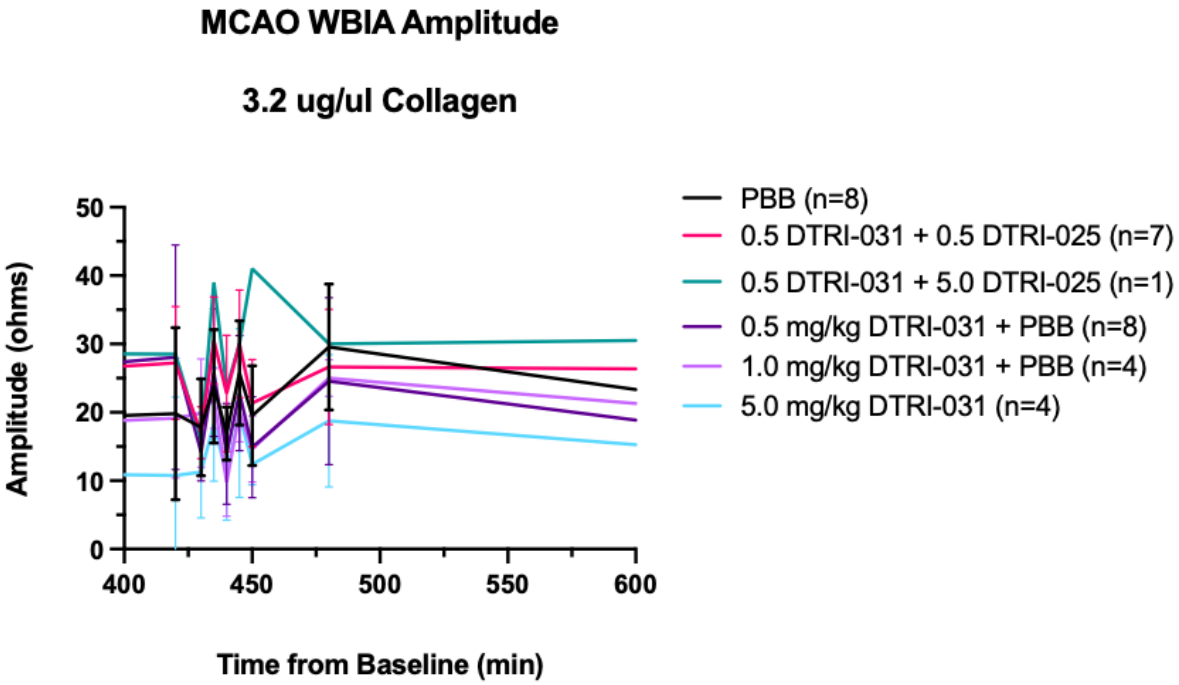



MCAO WBIA Slope
20 uM ADP
Slope (ohms/min)
20
15
10
5
0
400
450
500
550
600
Time from Baseline (min)
PBB (n=8)
0.5 DTRI-031 + 0.5 DTRI-025 (n=7)
0.5 DTRI-031 + 5.0 DTRI-025 (n=1)
0.5 mg/kg DTRI-031 + PBB (n=8)
1.0 mg/kg DTRI-031 + PBB (n=4)
5.0 mg/kg DTRI-031 (n=4)


MCAO WBIA Slope
3.2 ug/ul Collagen
Slope (ohms/min)
30
20
10
0
400
450
500
550
600
Time from Baseline (min)
PBB (n=8)
0.5 DTRI-031 + 0.5 DTRI-025 (n=7)
0.5 DTRI-031 + 5.0 DTRI-025 (n=1)
0.5 mg/kg DTRI-031 + PBB (n=8)
1.0 mg/kg DTRI-031 + PBB (n=4)
5.0 mg/kg DTRI-031 (n=4)


MCAO WBIA Amplitude
1 ug/ul Botrocetin
Amplitude (ohms)
60
40
20
0
400
450
500
550
600
Time from Baseline (min)
PBB (n=8)
0.5 DTRI-031 + 0.5 DTRI-025 (n=7)
0.5 DTRI-031 + 5.0 DTRI-025 (n=1)
0.5 mg/kg DTRI-031 + PBB (n=8)
1.0 mg/kg DTRI-031 + PBB (n=4)
5.0 mg/kg DTRI-031 (n=4)


MCAO WBIA Amplitude
20 uM ADP
Amplitude (ohms)
30
20
10
0
400
450
500
550
600
Time from Baseline (min)
PBB (n=8)
0.5 DTRI-031 + 0.5 DTRI-025 (n=7)
0.5 DTRI-031 + 5.0 DTRI-025 (n=1)
0.5 mg/kg DTRI-031 + PBB (n=8)
1.0 mg/kg DTRI-031 + PBB (n=4)
5.0 mg/kg DTRI-031 (n=4)


MCAO WBIA Amplitude
3.2 ug/ul Collagen
Amplitude (ohms)
50
40
30
20
10
0
400
450
500
550
600
Time from Baseline (min)
PBB (n=8)
0.5 DTRI-031 + 0.5 DTRI-025 (n=7)
0.5 DTRI-031 + 5.0 DTRI-025 (n=1)
0.5 mg/kg DTRI-031 + PBB (n=8)
1.0 mg/kg DTRI-031 + PBB (n=4)
5.0 mg/kg DTRI-031 (n=4)

VIII. Bibliography:

1. American Stroke Association. “About Stroke.” *Www.stroke.org*, 2021, www.stroke.org/en/about-stroke.
2. Nogles, Teryn. “Middle Cerebral Artery Stroke.” *Stat Pearls*, 2020.
3. Jilani, Talha. “Tissue Plasminogen Activator.” *Stat Pearls*, 2021.
4. Keefe, A., Pai, S. & Ellington, A. Aptamers as therapeutics. *Nat Rev Drug Discov* **9,** 537–550 (2010). https://doi.org/10.1038/nrd3141
5. Stroke Center. “The Internet Stroke Center.” *The Internet Stroke Center. An Independent Web Resource for Information about Stroke Care and Research.*, 2002, www.strokecenter.org/patients/about-stroke/stroke-statistics/#:~:text=Worldwide%20Statistics,than%2012.7%20million%20strokes%20worldwide.
6. Virani, Salim S., et al. “Heart Disease and Stroke Statistics—2021 Update: A Report From the American Heart Association.” *Circulation*, 2021, doi:10.1161/cir.0000000000000950.
7. Thachil, Jecko. “Antiplatelet Therapy – a Summary for the General Physicians.” *Clinical Medicine*, vol. 16, no. 2, 2016, pp. 152–160., doi:10.7861/clinmedicine.16-2-152.
8. Gartner, Arnold H. “Aspirin-Induced Gastritis and Gastrointestinal Bleeding.” *The Journal of the American Dental Association*, vol. 93, no. 1, 1976, pp. 111–117., doi:10.14219/jada.archive.1976.0591.
9. Gorelick, Philip B., and Steven M. Weisman. “Risk of Hemorrhagic Stroke With Aspirin Use.” *Stroke*, vol. 36, no. 8, 2005, pp. 1801–1807., doi:10.1161/01.str.0000174189.81153.85.

10. Hacke, W. “Most US Hospitals Don't Offer TPA to Ischemic Stroke... : Neurology Today.” *LWW*, 2009, journals.lww.com/neurotodayonline/fulltext/2009/05040/Most_US_Hospitals_Don_t_Offer_tPA_to_Ischemic.6.aspx.
11. Coverdell, Paul. “Acute Stroke Care in the US.” *Stroke*, vol. 36, no. 6, 2005, pp. 1232–1240., doi:10.1161/01.str.0000165902.18021.5b.
12. Marler, John. “Tissue Plasminogen Activator for Acute Ischemic Stroke.” *New England Journal of Medicine*, vol. 333, no. 24, 1995, pp. 1581–1588., doi:10.1056/nejm199512143332401.
13. Davis, C., et al. “The Role of Inflammation in Vascular Injury and Repair.” *Journal of Thrombosis and Haemostasis*, vol. 1, no. 8, 2003, pp. 1699–1709., doi:10.1046/j.1538-7836.2003.00292.x.
14. Varga-Szabo, David, et al. “Cell Adhesion Mechanisms in Platelets.” *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, no. 3, 2008, pp. 403–412., doi:10.1161/atvbaha.107.150474.
15. Conway, DS, Pearce, LA, Chin, BS, HART, RG and Lip, GY (2003). Prognostic value of plasma von Willebrand factor and soluble P-selectin as indices of endothelial damage and platelet activation in 994 patients with nonvalvular atrial fibrillation. *Circulation* **107:** 3141-3145
16. Kamphuisen, PW, Eikenboom, JC and Bertina, RM (2001). Elevated factor VIII levels and the risk of thrombosis. *Arterioscler Thromb Vasc Biol* **21:** 731-738
17. Nimjee SM. Rapidly regulating platelet activity in vivo with an antidote controlled platelet inhibitor. *Molecular therapy*. 02/2012;20(2):391-397. doi: 10.1038/mt.2011.226.

18. Denis CV, Wagner DD. Platelet adhesion receptors and their ligands in mouse models of thrombosis. Arterioscler Thromb Vasc Biol. 2007;27(2):728–739. [PubMed: 17272754]
19. Nimjee SM. The potential of aptamers as anticoagulants. *Trends in Cardiovascular Medicine*. 01/2005;15(1):41-45. doi: 10.1016/j.tcm.2005.01.002
20. Sheng Liu 2012 - A novel embolic stroke model resembling lacunar infarction following proximal middle cerebral artery occlusion in beagle dogs
21. Ruggeri ZM. The role of von Willebrand factor in thrombus formation. *Thromb Res*. 2007;120 Suppl 1(Suppl 1):S5-S9. doi:10.1016/j.thromres.2007.03.011
22. Figure 2. Self-Created by Cole Anderson
23. Cosmi, B (2009). ARC-1779, a PEGylated aptamer antagonist of von Willebrand factor for potential use as an anticoagulant or antithrombotic agent. Curr Opin Mol Ther 11: 322–328.
24. Diener, JL, Daniel Lagassé, HA, Duerschmied, D, Merhi, Y, Tanguay, JF, Hutabarat, R et al. (2009). Inhibition of von Willebrand factor-mediated platelet activation and thrombosis by the anti-von Willebrand factor A1-domain aptamer ARC1779. J Thromb Haemost 7: 1155–1162.
25. Wu, D, Vanhoorelbeke, K, Cauwenberghs, N, Meiring, M, Depraetere, H, Kotze, HF et al. (2002). Inhibition of the von Willebrand (VWF)-collagen interaction by an antihuman VWF monoclonal antibody results in abolition of in vivo arterial platelet thrombus formation in baboons. Blood 99: 3623–3628.
26. Yamamoto, H, Vreys, I, Stassen, JM, Yoshimoto, R, Vermylen, J and Hoylaerts, MF (1998). Antagonism of vWF inhibits both injury induced arterial and venous thrombosis in the hamster. Thromb Haemost 79: 202–210.

27. Cadroy, Y, Hanson, SR, Kelly, AB, Marzec, UM, Evatt, BL, Kunicki, TJ et al. (1994). Relative antithrombotic effects of monoclonal antibodies targeting different platelet glycoprotein-adhesive molecule interactions in nonhuman primates. Blood 83: 3218–3224.

28. Rumbaut, R.E. "Platelet-Vessel Wall Interactions in Hemostasis and Thrombosis." *Morgan and Claypool Lifesciences*, 2010.

29. Nimjee, Shahid M., et al. "Preclinical Development of a VWF Aptamer to Limit Thrombosis and Engender Arterial Recanalization of Occluded Vessels." *Molecular Therapy*, vol. 27, no. 7, 2019, pp. 1228–1241., doi:10.1016/j.ymthe.2019.03.016.

30. Chicago, IL, General Electric. *GE 9900 Fluoroscope*.

31. Joseph, Matthew. "Interventional Cardiology Core, ICC, OSUWMC." 2019, Columbus, OH, Wexner Medical Center.

32. Kan, I., et al. "A Novel Method of Thrombus Preparation for Use in a Swine Model for Evaluation of Thrombectomy Devices." *American Journal of Neuroradiology*, vol. 31, no. 9, 2010, pp. 1741–1743., doi:10.3174/ajnr.a1991.

33. Nimjee, SM, Povsic, T, Sullenger, BA, Becker, RC. Translation and clinical development of antithrombotic aptamers. *Nucleic Acid Therapeutics*. Vol. 26(3): 147-55, June 2016

34. Kumar V. Cotran RS, Robbins SL. *Basic Pathology*. 6th ed. Philadelphia: W.B. Saunders; 1997

35. Christoforidis GA, Rink C, Kontzialis MS, Mohammad Y, Koch RM, Abduljalil AM, Bergdall VK, Roy S, Khanna S, Slivka AP, Knopp MV, Sen CK. 2011. An endovascular canine middle cerebral artery occlusion model for the study of leptomeningeal collateral recruitment. Invest Radiol. 2011 Jan;46(1):34-40.

36. Qureshi AI, Boulos AS, Hanel RA, Suri MF, Yahia AM, Alberico RA, Hopkins LN. Randomized comparison of intra-arterial and intravenous thrombolysis in a canine model of acute basilar artery thrombosis. *Neuroradiology.* 2004;46(12):988-995.
37. Hacking, Craig. "Thrombolysis in Cerebral Infarction (TICI) Scale: Radiology Reference Article." *Radiopaedia Blog RSS*, 2013, radiopaedia.org/articles/thrombolysis-in-cerebral-infarction-tici-scale?lang=us.
38. Thiagarajan, Perumal. "What Is the Platelet Function Analyzer 100 (PFA-100) and How Is It Used in the Workup of Platelet Disorders?" *Latest Medical News, Clinical Trials, Guidelines - Today on Medscape*, 13 Dec. 2019, www.medscape.com/answers/201722-90211/what-is-the-platelet-function-analyzer-100-pfa-100-and-how-is-it-used-in-the-workup-of-platelet-disorders.
39. Medicine, Sang. "Practical-Haemostasis.com." *Platelet Function Testing: Impedance Platelet Aggregometry*, 2021, www.practical-haemostasis.com/Platelets/platelet_function_testing_impedance_platelet_aggregometry.html.
40. "Accriva Diagnostics." *DAIC*, 17 May 2016, www.dicardiology.com/company/accriva-diagnostics.